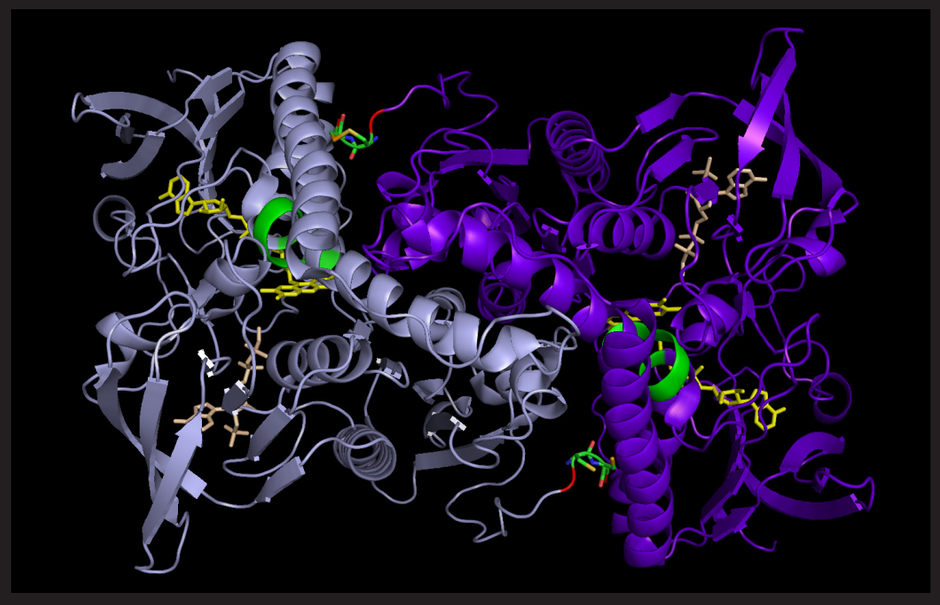

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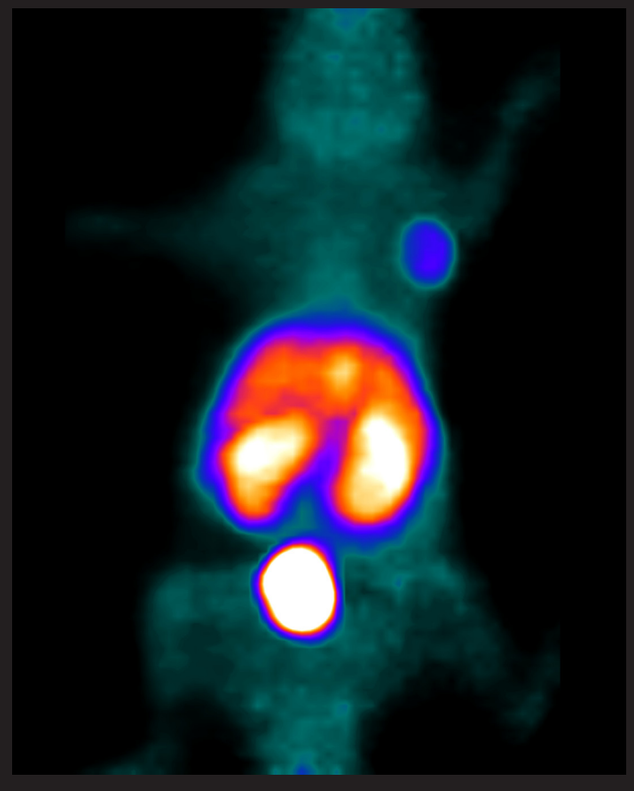

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# DEVELOPMENT OF A SEL-TAG FOR MULTIMODALITY IMAGING AND STUDIES OF MAMMALIAN THIOREDOXIN REDUCTASE 1

Qing Cheng


Stockholm 2010

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***Front cover:*** PET imaging of SCID mouse bearing xenograft tumor developed from FaDu cells (squamous cell carcinoma of pharynx, *Homo sapiens*) utilizing carbon-11 labeled Sel-tagged annexin A5. Tumor was located at the animal's right shoulder. High radioactivity uptake in the liver, kidney and bladder was also observed.

***Back cover:*** Crystal structure of rat Thioredoxin Reductase 1 with enzyme's active sites and cofactors highlighted

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Printed by Larserics Digital Print AB, Sweden
Published by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

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**ISBN 978-91-7457-020-5**

**IN MEMORY OF MY FATHER**

**TO MY FAMILY**

致我的家人并谨此纪念我的父亲

*“I love fools' experiments. I am always making them.”*

**Charles R. Darwin**

(12 February 1809 - 19 April 1882)

*“A tidy laboratory means a lazy chemist.”*

**Jöns Jacob Berzelius**

(20 August 1779 - 7 August 1848)

# ABSTRACT

Selenocysteine (Sec; U in one-letter code), the 21st amino acid existing in all selenoproteins, has unique biochemical properties due to its selenium atom, including a low p*K*a and a high reactivity with many electrophilic agents. When a selenoprotein is translated, the insertion of Sec occurs at a UGA codon, normally yielding translational termination. Here UGA can be re-coded as Sec through the presence of a species-specific downstream mRNA sequence called Sec insertion sequence (SECIS) element. As one of about 25 mammalian selenoproteins, thioredoxin reductase 1 (TrxR1) contains one Sec residue within its C-terminal tetrapeptide motif, -Gly-Cys-Sec-Gly-COOH. By utilizing an engineered bacterial SECIS element in the rat TrxR1 cDNA, we are able to produce recombinant rat TrxR1 with both high yields and specific activities, which gives us an international leading edge, as selenoprotein production is a common bottleneck for researchers in the field of selenoprotein biology. Based on the studies on TrxR1, we have developed a "Sel-tag" for recombinant proteins using the C-terminal tetrapeptide motif of TrxR1. We have subsequently demonstrated that the selenolate of a reduced Sel-tag provides a "handle" which can be utilized for a wide range of selenolate-targeted applications, including a one-step protein purification procedure, residue-specific fluorescent labeling and radiolabeling with either gamma emitters ($^{75}$Se) or positron emitting radionuclides ($^{11}$C).

We first utilized Sel-tag to investigate the mechanism of antigen presentation through carbohydrate based particles (CBP) by tracking the selenium-75 labeled Sel-tagged Fel d 1, the major cat allergen, in a mouse model.

We further applied this technique for studies of apoptosis with a particular interest in utilizing Sel-tagged annexin A5 to accomplish multimodality imaging. We demonstrate that Sel-tagged annexin A5 labeled either with fluorescent 5-iodoacetamidofluorescein (5-IAF) or the positron emitter carbon-11 remains functional and specifically binds apoptotic cells. This proved to be useful for (i) utilizing fluorescently labeled annexin A5 in either the conventional annexin A5 affinity assay with flow cytometry or in fluorescent microscopy of cells undergoing cell death, (ii) PET imaging of apoptotic liver of health BALB/c mice treated with anti-FAS antibody, and (iii) PET imaging of xenograft tumors developed from FaDu cells in SCID mice.

We have solved the crystal structure of wild type rat TrxR1 and probed its catalytic mechanism. We have shown that the oxidized protein presents a stabilized selenenylsulfide motif in *cis*-configuration, with the Sec residue coordinated to the planar surface of a tyrosine side chain (Tyr116), thus located far from the redox active moieties of the enzyme proposed to be involved in electron transport to the C-terminal motif during catalysis. We believe that Tyr116 plays an important role for catalysis of TrxR1, possibly by involvement in electron transfer during the reductive half reaction, or by stabilizing the selenenylsulfide configuration of oxidized TrxR1.

It has been shown that TrxR1 has pro-oxidant roles especially upon redox cycling with certain low molecular weight substrates. Thus we have investigated the potential of ROS generation by TrxR1 using wild type enzyme as well as several TrxR1 mutants. Utilizing Electron Spin Resonance (ESR) spin trapping with 5-Diethoxyphos-phoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), we found that TrxR1 could generate free radicals including both hydroxyl radicals ($HO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) upon NADPH reduction in the absence of other substrates, and the $HO^{\cdot}$ generation was largely dependent upon prior enzyme-catalyzed $O_2^{\cdot -}$generation, indicating an inherent peroxidase activity of TrxR1. Closer analyses revealed that the DEPMPO/$HOO^{\cdot}$ adduct was a direct substrate of TrxR that could be reduced to DEPMPO/$HO^{\cdot}$ in a Sec-dependent manner.

# LIST OF PUBLICATIONS

***Articles included as the basis of this thesis***

**I.** **Cheng Q**, Johansson L, Thorell J-O, Fredriksson A, Samén E, Stone-Elander S, Arnér ESJ. Selenolthiol and dithiol C-terminal tetrapeptide motifs for one-step purification and labeling of recombinant proteins produced in *E. coli*. ChemBioChem. 2006; 7(12):1976-1981

**II.** Thunberg S*, Neimert-Andersson T*, **Cheng Q**, Wermeling F, Bergström U, Swedin L, Dahlén SE, Arnér ESJ, Scheynius A, Karlsson MC, Gafvelin G, van Hage M, Grönlund H. Prolonged antigen-exposure with carbohydrate particle based vaccination prevents allergic immune responses in sensitized mice. Allergy. 2009; 64(6):919-926
* Both authors contributed equally to this work

**III.** **Cheng Q**, Thorell J-O, Lu L, Samén E, Hägg-Olofsson M, D'Arcy P, Ahlzén H-S, Linder S, Stone-Elander S, and Arnér ESJ. Multimodality imaging of Apoptosis through Sel-tagged Annexin A5. Manuscript

**IV.** **Cheng Q**, Sandalova T, Lindqvist Y, Arnér ESJ. Crystal structure and catalysis of the selenoprotein thioredoxin reductase 1. Journal of Biological Chemistry. 2009 284(6): 3998-4008

**V.** **Cheng Q***, Antholine WE*, Myers JM, Kalyanaraman B, Arnér ESJ, Myers CR. The selenium-independent inherent pro-oxidant NADPH oxidase activity of mammalian thioredoxin reductase and its selenium-dependent direct peroxidase activities. Journal of Biological Chemistry. 2010; 285(28):21708-21723
* Both authors contributed equally to this work

***Article included in this thesis as Appendix***

**Cheng Q**, Stone-Elander S, Arnér ESJ. Tagging recombinant proteins with a Sel-tag for purification, labeling with electrophilic compounds or radiolabeling with $^{11}$C. Nature Protocols. 2006;1(2):604-613.

***Articles not included in this thesis***

Rengby O, **Cheng Q**, Vahter M, Jörnvall H, Arnér ESJ. Highly active dimeric and low-activity tetrameric forms of selenium-containing rat thioredoxin reductase 1. Free Radical Biology and Medicine. 2009 Apr 1;46(7):893-904

Stenvall J, Fierro-González JC, Swoboda P, Saamarthy K, **Cheng Q**, Cacho-Valadez B, Arnér ESJ, Persson OP, Miranda-Vizuete A, Tuck S. The selenoprotein Thioredoxin Reductase 1 and Glutathione Reductase are essential for removal of old cuticle during molting in *C. elegans*. Manuscript submitted.

Rackham O, Shearwood AMJ, Thyer R, McNamara E, Davies SMK, Callus BA, Miranda-Vizuete A, Berners-Price SJ, **Cheng Q**, Arnér ESJ, Filipovska A. Difference in substrate preferences and metal compound inhibition of cytosolic and Mitochondrial human thioredoxin reductases. Manuscript submitted.

Prast-Nielsen P, Dexheimer T, Schultz L, **Cheng Q**, Xu J, Jadhav A, Arnér ESJ, Simeonov A. Inhibition of thioredoxin reductase 1 by protoporphyrin IX identified with a novel high throughput screening assay. Manuscript submitted.

# TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| [$^{18}$F]FDG | [$^{18}$F] fluorodeoxyglucose |
| 3'-UTR | 3'-untranslated region |
| 5-IAF | 5-Iodoacetamidofluorescein |
| Alum | Aluminum hydroxide |
| AnxA5 | Anneixn A5 |
| CBP | Carbohydrate based particles |
| Cys | Cysteine |
| DEPMPO | 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide |
| DIO | Iodoththyronine deiodinase |
| DMPS | 2,3-dimercapto-1-propanesulfonic acid, sodium salt |
| DTNB | 5, 5′-dithiobis-(2-nitrobenzoic acid) or Ellman's reagent |
| DTT | Dithiothreitol |
| ESR | Electron spin resonance |
| EU | Endotoxin Units |
| FAD | Flavin adenine dinucleotide |
| FDH | Formate dehyrogenase |
| Fel d 1 | Major cat allergen |
| fMet | *N*-Formylmethionine |
| Gly | Glycine |
| GPx | Glutathione peroxidase |
| GSH | Glutathione |
| GSSG | Glutathione disulfide |
| IPTG | Isopropyl-*β*-D-thiogalactose |
| Juglone | 5-hydroxy-1,4-naphthoquinone |
| LPS | Lipopolysaccaride |
| mAb | Monoclonal antibody |
| Met | Methionine |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced) |
| $O_2^{\cdot -}$ (or $HOO^{\cdot}$) | Superoxide |
| $OH^{\cdot}$ | Hydroxyl radical |
| ORF | Open reading frame |
| PAO | Phenylarsine oxide |
| PET | Positron emission tomography |
| Pyl | Pyrrolysine |
| Sec (or U) | Selenocysteine |
| SECIS | Sec insertion sequence |
| SelA | *E. coli* Sec synthase |
| SelB | *E. coli* Sec-specific elongation factor |
| SelC | *E. coli* Sec specific tRNA |
| SelD | *E. coli* selenophosphate synthetase |
| SerS | *E. coli* seryl-tRNA synthetase |
| SOD | Superoxide dismutase |
| SPS2 | Mammalian selenophosphate synthetase 2 |
| Trx | Thioredoxin |
| TrxR | Thioredoxin reductase |

# 1 INTRODUCTION

## 1.1 Selenium

### 1.1.1 General introduction

Selenium was discovered in 1817 by Swedish chemists Jöns Jakob Berzelius (1779-1848) and Johan Gottlieb Gahn (1745-1818) as a byproduct of sulfuric acid production originally from the copper mine in Falun, Sweden. It was originally thought to be the element tellurium that had been discovered thirty years previously, which was later on proved to be a mistake after careful analysis by Berzelius. Nonetheless, in relation to tellurium which was named from the Latin word *tellus* for "Earth", Berzelius named the new element selenium, from the Greek *selene* (Σελήνη) meaning "Moon" [1].

Selenium is a rare element ranking among the 25 least common elements in the earth's crust. The standard atomic mass of selenium is 78.96 and there are six naturally occurring isotopes of selenium: $^{74}$Se (0.87%), $^{76}$Se (9.36%), $^{77}$Se (7.63%), $^{78}$Se (23.78%), $^{80}$Se (49.61%) and $^{82}$Se (8.73%). The first five are stable isotopes, and $^{82}$Se has a half life of around $10^{20}$ years, which therefore could also be considered as stable isotope for practical purpose [2]. More than dozens of radioactive isotopes of selenium have been characterized. Among them, $^{75}$Se is an artificial radioactive selenium isotope which has been widely used in the clinic and in research [3; 4; 5; 6; 7], including this thesis.

### 1.1.2 Selenium in biology

For a very long time after selenium had been discovered and widely utilized in engineering, the major biological property of selenium was considered to be its toxicity. Today we know that selenium toxicity is different among individuals and many factors could contribute, such as the dose, type of selenium and the exposure time. Symptoms of selenium poisoning include significant hair loss, dermatitis, paralysis, muscular problems, nausea, fatigue, diarrhea, skin inflammation, etc [8; 9; 10]. So far, no proven antidote for selenium poisoning has been reported [11]. On the other hand, it wasn't until the middle of the last century that selenium was recognized to be essential to life [12; 13]. Selenium deficiency contributes to several diseases in animals [14; 15; 16], and the first human disease shown to be caused by selenium deficiency was Keshan disease reported in 1979 [17].

In mammals, selenium plays an important role in protecting tissues from oxidative stress as a component of glutathione peroxidase, which catalyzes the reduction of lipid hydroperoxides and free hydrogen peroxide to their corresponding alcohols or water, and in thioredoxin reductase, which catalyzes the NADPH-dependent reduction of thioredoxin [18; 19]. It is also found in the deiodinases, which convert thyroxine to triiodothyronine [20]. The biologically active form of selenium in these enzymes is a naturally occurring amino acid, selenocysteine.

## 1.2 Selenocysteine

### 1.2.1 Chemical properties of selenocysteine

Chemically, selenium shares a lot of similarities with sulfur, and differs only slightly in properties such as in their ionic radius, electronegativity, covalent radius, oxidation state etc [21].

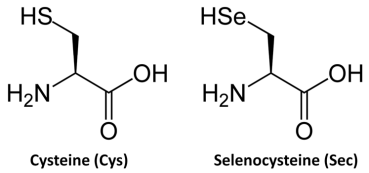



**Figure 1. Comparison of cysteine and selenocysteine.**

Selenocysteine (Sec; U in the one-letter code) is often referred as the 21st naturally occurring amino acid [22]. As the name suggested, it is structurally similar to cysteine (Cys), with an atom of selenium taking the place of the sulfur, forming a selenol moiety (Figure 1). Sec has unique biochemical properties due to its selenium atom, including a low p*K*a and a high reactivity with many electrophilic agents [23].

### 1.2.2 Expansion of genetic code

The standard genetic code contains 64 codons as shown in most textbooks and literature. 61 codons was designated for the common 20 amino acids and 3 codons for signaling the termination of protein translation [24]. However, more and more studies have revealed that certain codons are dual functional, especially the codons designated for protein translation termination [25]. There are two amino acids, among many others than the twenty that are part of the genetic code, that have been discovered to be coded genetically: Sec and pyrrolysine (Pyl). Sec is coded by the stop codon UGA (umber) by species-specific mechanisms dependent on structural elements of the mRNA (see

section 1.3). Pyl is considered to be the 22nd naturally occurring amino acid identified only in a few archaea and bacteria, is encoded by UAG, which also serves as stop codon (amber) in most organisms [26]. It is worthy of mention that in ciliate *Euplotes crassus*, the codon UGA is used for insertion of both Sec and Cys, even within the same genes [27]. And in mycoplasma species, UGA is used for tryptophan insertion as well as termination [28]. The codon AUG is also considered to be dual functional, for initiating protein translation encoding methionine (Met) in eukaryotes or *N*-formylmethionine (fMet) in prokaryotes, and for encoding internal Met within the protein [25].

## 1.3 Selenoprotein

### 1.3.1 General introduction

Selenoprotein exclusively refers to proteins that include one or several selenocysteine residues, although there are other selenium modified amino acids such as selenomethionine, which may be incorporated into protein through non-genetic pathways [29]. Selenoproteins are found in all three domains of life. In eukaryote, selenoproteins exist almost exclusively in animals. Several selenoproteins have been identified in the green alga *Chlamydomonas*. No selenoproteins have yet been indentified in fungi or plants [30]. Among bacteria and archaea, selenoproteins seem to be less common compared to eukaryotes and only exist in some phylogenetic groups [31].

In recent years, several bioinformatics studies performed by Gladyshev and coworkers have extensively broadened our knowledge about selenoprotein distributions in different species [32]. There are 25 selenoproteins from human, 24 from rodent [33], at least 15 different types of selenoproteins from bacteria and archaea, one single selenoprotein (cytosolic thioredoxin reductase) from *C. elegans* [34; 35]. In addition, more than 300 different selenoproteins genes were found in samples from the Sargasso Sea [36] and more than 3600 selenoprotein genes derived from 58 selenoprotein families were identified based upon the Global Ocean Sampling (GOS) expedition [37].

Specifically in *E. coli*, three selenoproteins have been identified, all of which are formate dehydrogenases (FDH) expressed under different conditions. $FDH_G$ is synthesized in aerobic conditions while $FDH_F$ and $FDH_N$ are only synthesized in anaerobic cells [38]. In archaea, a few dehydrogenases and hydrogenases as well as glycine reductase were identified as selenoproteins [31; 39]. In eukaryote, the types of

selenoproteins are very different from those in lower organisms with almost no overlap. Mammalian selenoproteins will be described in the next section.

### 1.3.2 Mammalian selenoproteins

Mammalian selenoproteins can be generally categorized into two groups, one group with Sec closely located to the C-terminus, often in the penultimate position. In the other group Sec is located in the N-terminal domain, often in a thioredoxin fold with redox activity and functionality [33; 40]. The most studied selenoproteins in mammals are several types of selenoenzymes that include glutathione peroxidases (GPxs), thioredoxin reductases (TrxRs), and iodothyronine deiodinases (DIOs).

GPx was first discovered in 1957 [41] and first identified as a selenoprotein in 1973 [6; 18]. There are five GPxs in humans and four in rodents that are selenoproteins, among some other non-selenoprotein GPx family members [33]. As mentioned in section 1.1.2, GPxs reduce hydroperoxide or free $H_2O_2$ to the corresponding alcohol or water, using glutathione (GSH) as cofactor, which makes this enzyme an important antioxidant enzyme. In the GPx Sec is normally present in its highly reactive deprotonated selenolate form (-$Se^-$), which can be oxidized by peroxides to selenenic acid (-SeOOH). The peroxide is therefore reduced to its alcohol form (or water if the substrate is $H_2O_2$). The selenenic acid in GPx is, however, reduced by two GSH molecules to form selenolate again, releasing a glutathione disulfide (GSSG) molecule as the byproduct. The oxidized GSSG is subsequently reduced to GSH again by glutathione reductase in the presence of NADPH to complete the catalytic cycle. Historically, cytosolic GPx (GPx1) was also the first identified mammalian selenoprotein. More details about GPxs including different isoforms and enzymatic catalysis as well as the function of the enzyme can be found in these references [42; 43; 44; 45].

Mammalian TrxRs catalyze the NADPH-dependent reduction of the redox-active disulfide in thioredoxin (Trx), which serves a wide range of functions in cellular proliferation and redox control [46; 47; 48]. In addition to the principle substrate Trx, TrxRs react with a broad range of other substrates including low molecular weight disulfide-containing compounds (e.g. DTNB, lipoamide and lipoic acid), inorganic selenium-containing compounds (e.g. selenite), organic selenium compounds (e.g. ebselen) and some quinones (e.g. juglone) [49; 50; 51; 52]. More details about this selenoenzyme will be described in section 1.4.3.

Mammalian DIOs include three isoforms, DIO1, DIO2, DIO3, which are involved in thyroid hormone activation and inactivation [53]. DIO1 and DIO2 catalyze the conversion of the prohormone, thyroxine (T4), to the active hormone, 3,5,3'-triiodothyronine (T3). DIO3 comprises the major inactivating pathway that terminates the action of T3 and prevents the activation of T4 [54]. More details about the DIOs are given in these references [55; 56; 57].

It is also noteworthy that selenoprotein P (SELP) is the only known selenoprotein containing multiple Sec within one polypeptide. Human SELP contains 10 Sec residues, with a longer N-terminal domain containing a single Sec in a redox Trx fold and a shorter C-terminal domain containing the remaining 9 Sec residues. SELP's expression, function and role in mammals have recently been reviewed [58; 59].

Other mammalian selenoproteins include selenophosphate synthetase 2 (SPS2), which is a selenoprotein that is also involved in other selenoproteins' biosynthesis [60], and a few selenoproteins whose functions are completely or partially unknown, such as selenoprotein H, I, K, M, N, O, R, S, T, V, W and a 15 kDa selenoprotein [33; 61; 62; 63; 64; 65; 66].

## 1.4 Thioredoxin system

### 1.4.1 General introduction

The Trx system comprises Trx, TrxR and NADPH and is widely distributed in almost all living organisms. The *E. coli* Trx system was discovered by Peter Reichard and coworkers in 1964 as a hydrogen donor for the enzymatic synthesis of deoxyribonucleotides by ribonucleotide reductase (RNR) [67]. The major difference between bacterial and mammalian Trx systems is that mammalian TrxR exists as a selenoprotein as mentioned earlier. The Trx system, together with the glutaredoxin (Grx) system, plays a vital role in maintaining the redox state of the cell and protecting the cell against oxidative stress by catalyzing the reduction of their corresponding substrates, especially protein substrates containing a disulfide (Figure 2) [68; 69].

### 1.4.2 Thioredoxin

Trx is a 12-kDa oxidoreductase containing a dithiol/disulfide active site. It is found in almost all organisms from plant, bacterial, archaea to mammals. Different isoforms of Trx are found in most organisms [70; 71; 72; 73]. In mammals, two Trx isoforms have been identified, namely cytosolic Trx (Trx1) and mitochondrial Trx (Trx2). Both

isoforms are essential for mammals [74; 75]. Trx belongs to a protein family with a common structure termed Trx fold [76]. The sequence similarity among Trxs is relatively high across different organisms and they share a common active site sequence with two vicinal Cys in a CXXC motif [77]. Trx's substrates are broad including ribonucleotide reductase, GPx, peroxiredoxin (Prx), insulin, $H_2O_2$, GSSG, etc. Insulin is a classic substrate used in Trx activity assays [46; 78].

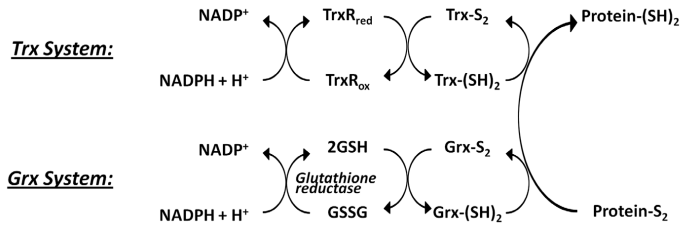



**Figure 2. Redox reactions catalyzed by the Trx and Grx systems.** NADPH as the electron donor for both systems is mainly produced from the pentose phosphate pathway. Both systems share certain common functions, however, their specific roles could be different in each organism under different circumstances [48].

### 1.4.3 Thioredoxin reductase

TrxRs are dimeric flavoenzymes belonging to the pyridine nucleotide-disulfide oxidoreductase family, which is present in all living organisms [79; 80]. Two TrxR forms have evolved independently and they are mostly distinguished by their sizes of 70kDa and 110kDa as dimers. The low-$M_r$ TrxRs are found in lower organisms such as bacteria, fungi as well as in plants, while the high-$M_r$ TrxRs are mostly found in higher eukaryotes [47]. Both types of TrxRs contain one FAD and one redox active disulfide in each subunit using NADPH as the co-enzyme. However, the high-$M_r$ TrxRs also have a third active site located in each subunit's C-terminal as a -XCUX or -XCCX motif. The TrxRs containing the selenenylsulfide/selenolthiol motif (-XCUX) exist in mammals, while those containing the disulfide/dithiol motif (-XCCX) are found in insects such as *D. melanogaster* [81] and *A. gambiae* [82]. In lower eukaryotes such as *P. falciparum,* the third active site exists as a -CXXXXCG motif [83]. The catalytic mechanism of these two types of TrxR are also different and the high-$M_r$ TrxRs have a much broader substrate spectrum mainly, if not all, due to their highly accessible C-terminal active site upon reduction.

Mammalian TrxR (EC 1.8.1.9) is a ubiquitous NADPH-dependent flavoenzyme that has prominent disulfide reductase activity. The cytosolic (TrxR1) and mitochondrial (TrxR2) isoforms are the only proteins known to reduce their respective Trx substrates (Trx1 and Trx2).

Mammalian TrxR1 (bovine) was first purified in 1977 [84], but it was not until 1995 that the enzyme was identified as a selenoprotein [19; 85]. It is difficult to study the use of the Sec residue in a selenoprotein from a structural perspective because of the limited supply of pure selenoprotein. Direct purification of native selenoproteins from their tissue or organ origins is very laborious due to the low abundance of selenoprotein. The barriers of selenoprotein biosynthesis machineries have also made structural studies on this unique type of protein difficult. Very few structures of native selenoproteins have yet been determined, including only two GPx isoforms purified from either bovine erythrocytes [86] or human plasma [87], and rat TrxR1 (**Paper IV**) [88], although several recombinant Sec mutants of different selenoproteins have been used to resolve the overall structure of this unique type of proteins [89; 90].

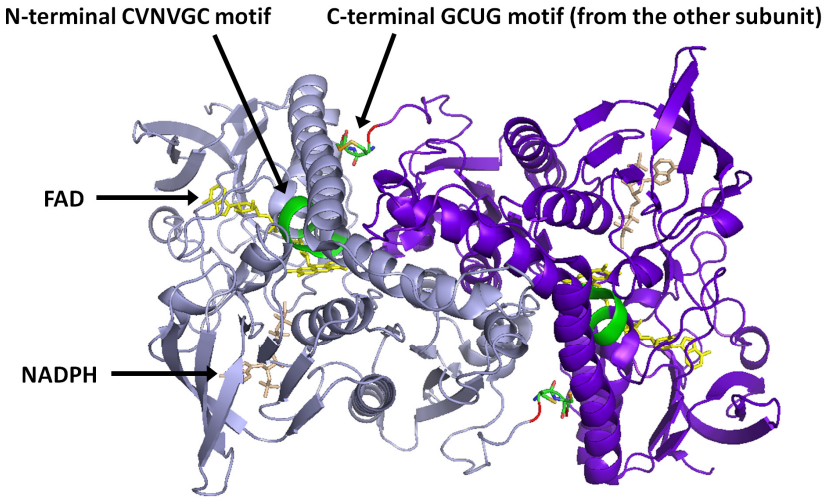



**Figure 3. An overall view of native rat TrxR1.** This view was built upon the atomic coordinates of the oxidized enzyme (PDB code 3EAO) by PyMOL [91], the active sites and co-enzyme are indicated by arrows.

The overall structure of mammalian TrxR is exhibited as a NADPH-dependent homodimer arranged in a head to tail orientation and each subunit contains three redox-active sites including one enzyme-bound FAD, one N-terminal -CVNVGC- motif and

one -GCUG motif existing in the very C-terminal of the enzyme (Figure 3). The enzymatic reaction catalyzed by mammalian TrxR is intricate with many factors and several active sites involved. Upon catalysis, a pair of electrons is transferred from NADPH via the FAD to the oxidized N-terminal disulfide to form a reduced dithiol motif. It subsequently exchanges the two electrons with the C-terminal selenenylsulfide to form a reduced selenolthiol motif [88; 92]. The reduced C-terminal selenolthiol is essential for the Sec-dependent reduction of oxidized Trx and most of the enzymes' substrates. However the reduction of some substrates, such as certain quinones, is Sec-independent and it's believed the N-terminal Cys59/Cys64 dithiol motif is responsible for that catalytic activity [93]. Figure 4 shows a detailed catalytic cycle of mammalian TrxR1.

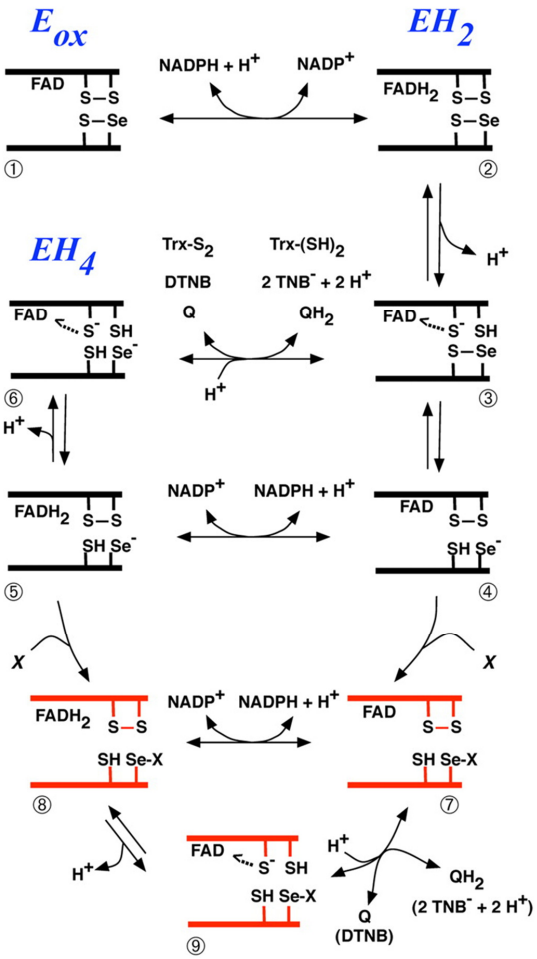


**Figure 4. Scheme of mammalian TrxR catalysis.** Oxidized enzyme (Eox, species 1) is reduced by NADPH to the two-electron reduced enzymes which are equilibrated among three different forms (EH2, species 2-4). The charge-transfer complex is indicated by a dashed arrow (species 3). The enzyme is further reduced by a second equivalent of NADPH to form a four-electron reduced enzyme (EH4, species 5 and 6). The second EH4 specie (6), containing a C-terminal selenolthiol motif as well as a charge-transfer complex, is believed to be the form of the enzyme normally reducing its substrates. A selenenylsulfide (in species 3) is formed subsequently upon catalysis (the oxidative half-reaction). The Sec-dependent catalysis is thus believed to occur with cycling of species 3 to 6. When the selenolate of the C terminus becomes exposed in the reduced enzyme, this can also be easily derivatized with electrophilic agents (X in the scheme), forming enzyme species 7 that is irreversibly inhibited for Sec-dependent reduction of substrates such as thioredoxin. However, NADPH can still reduce the FAD moiety to form species 9 via species 8. The inhibited enzyme species 9 can promote Sec-independent reduction of some substrates such as the quinone juglone (Q in the scheme) with high efficiency, or at about 5–10% efficiency compared with the native enzyme using DTNB as substrate.

Upon reduction of the enzyme, the nucleophilic and highly reactive selenolate form of the Sec residue is also prone to attack by electrophilic compounds as irreversible inhibitors, including some gold compounds such as auranofin [94], aurothioglucose [95], platinum compounds such as cisplatin [96] and oxaliplatin [97], nitrosoureas such as BCNU [98], dinitrohalobenzenes [99], arsenic oxide [100] and many more.

### 1.5 Selenoprotein biosynthesis

The biosynthesis of selenoproteins is a very complex process with many factors involved [101; 102; 103; 104]. As mentioned earlier, when a selenoprotein is translated, the insertion of Sec occurs at a UGA codon, normally resulting in translational termination. This expansion of the genetic code is intricate and requires a stem-loop structure in the mRNA, which is called the SECIS (Sec Insertion Sequence) element [105; 106]. The selenoprotein synthesis machinery can be generally divided into two categories. Eukaryote and archaea have similar machinery in terms of Sec synthesis and subsequent incorporation into selenoprotein. Bacteria have a very different pathway, especially in term of the location of SECIS in the selenoprotein mRNA. In bacterial selenoprotein mRNA, a SECIS element is situated next to the Sec-encoding UGA codon [107; 108]. In eukaryotes and archaea, on the other hand, SECIS elements are found

in the 3' untranslated region (3'-UTR) of the selenoprotein mRNA, which can direct single or multiple UGA codons for Sec insertion (Figure 5) [39; 109]. These significant differences in the selenoprotein biosynthesis machineries among bacteria, eukaryotes and archaea made the conventional techniques of heterologous recombinant protein expression in bacteria inapplicable for mammalian selenoprotein for a very long time, until the late 1990's [110; 111]. The success in recombinantly expressing mammalian TrxR in *E. coli* is the very foundation of this thesis, which will be described in the following.

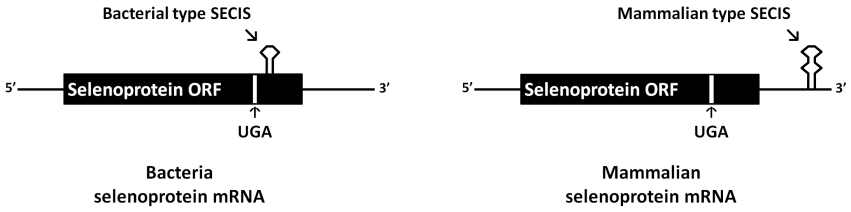



**Figure 5. Schematic comparison of the SECIS location in bacterial and mammalian selenoprotein mRNA.**

### 1.5.1 Selenoprotein expression in bacteria

The biochemical pathway of Sec synthesis and incorporation in bacteria was first elucidated by Böck and coworkers in the late 1980's [22]. Due to its high reactivity, Sec does not occur as a free amino acid and as a result there is no Sec pool for selenoprotein expression in the cell. Instead, cells use the less reactive selenide ($Se^{2-}$) as the selenium donor, and selenide is converted to selenophosphate by selenophosphate synthetase (SelD) [112]. Sec synthesis occurs on a dedicated transfer RNA, $tRNA^{Sec}$ (SelC) [113], which is different from standard tRNAs in several aspects. It has a longer extra arm of the variable loop, an 8-base pair aminoacyl-acceptor helix compared to the classic 7-base pair length, and substitutions at several well-conserved base positions (Figure 6) [114]. $tRNA^{Sec}$ is also the sixth serine (Ser) isoacceptor [102].

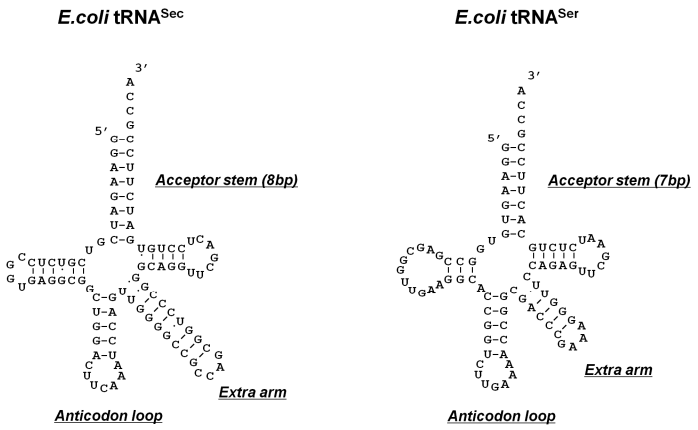



**Figure 6. Comparison of *E. coli* tRNA$^{Sec}$ and tRNA$^{Ser}$** [115].

During the biosynthesis of Sec, tRNA$^{Sec}$ is first charged with Ser by seryl-tRNA synthetase (SerS) to form Ser-tRNA$^{Sec}$, which is subsequently converted to Sec-tRNA$^{Sec}$ by selenocysteine synthase (SelA) using selenophosphate as the selenol group donor to replace the hydroxyl group in the tRNA$^{Sec}$ bound Ser (Figure 7).

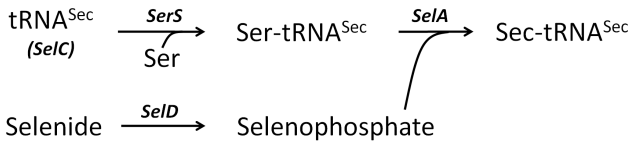



**Figure 7. Scheme of the Sec biosynthesis in bacteria**

Due to structural differences, neither Ser-tRNA$^{Sec}$ nor Sec-tRNA$^{Sec}$ is recognized by the *E. coli* elongation factor Tu (EF-Tu) during translation, which avoids unspecific Ser insertion into the growing polypeptides. Instead, *E. coli* has a dedicated elongation factor termed SelB for Sec incorporation [116; 117]. SelB contains two domains including the N-terminal SelB-N and C-terminal SelB-C. The SelB-N is homologous to EF-Tu, which specifically recruits Sec-tRNA$^{Sec}$ and GTP, while the SelB-C binds to the bacterial SECIS element. The formation of this quaternary complex of SelB, GTP, Sec-tRNA$^{Sec}$ and the SECIS element are required for decoding the UGA from the selenoprotein mRNA in bacteria (Figure 8) [102; 106].

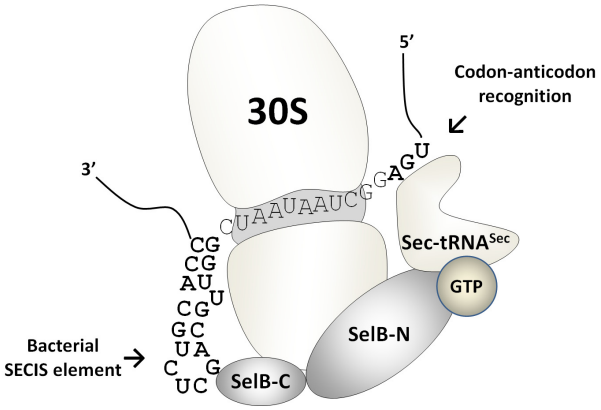



**Figure 8. Quaternary complex for Sec insertion in *E. coli*.** Sec incorporation occurs when the UGA codon is positioned at the ribosomal A site. A simultaneous recognition of the SECIS mRNA hairpin and the UGA codon by SelB charged with Sec-tRNA$^{Sec}$ is needed. Adapted from Ose (2007) [118].

The machinery of Sec incorporation is highly specific and precise to maintain selenoprotein syntheses. All the components involved have to be highly accurate and coordinate. For example, SerS must be able to use not only tRNA$^{Ser}$ but also tRNA$^{Sec}$ as the substrate to provide the precursor for Sec synthesis; the SelA must only convert Ser-tRNA$^{Sec}$ instead of Ser-tRNA$^{Ser}$ to avoid unspecific Sec insertion at a Ser codon; SelB must discriminate Sec-tRNA$^{Sec}$ from its precursor Ser-tRNA$^{Sec}$ and reject all standard tRNAs while the EF-Tu must reject tRNA$^{Sec}$ to ensure that Sec is precisely inserted at the UGA codon. Furthermore, the balance among SelB, Sec-tRNA$^{Sec}$ and the SECIS element is important for ensuring the formation of the correct quaternary complex for Sec incorporation. Most intriguingly, the level of SelB is regulated by a SECIS-like segment as a gene expression element on its own mRNA. To conclude, the bacterial selenoprotein synthesis is strictly regulated on many levels, including genetic, biochemical and physiological. See reference [102] for a complete review on this subject.

### 1.5.2 Selenoprotein expression in eukaryote

Although the selenoproteins expressed in eukaryotes and bacteria are almost completely independent, the mechanisms for Sec synthesis and incorporation are conserved in these two to a certain degree. For example, the biosynthesis of Sec in eukaryote also occurs on the dedicated transfer RNA, tRNA$^{Sec}$, and uses Ser-tRNA$^{Sec}$

as the precursor. The Sec incorporation in eukaryotes requires a SECIS element in the selenoprotein mRNA as well as a Sec-specific elongation factor. However, recent studies have revealed that eukaryotic selenoprotein biosynthesis has evolved to a higher level with many more factors and regulators involved [104].

As mentioned above, the most distinct feature of eukaryotic selenoprotein synthesis compared to bacteria is the location of the SECIS element. The fact that the eukaryotic SECIS is located in the 3'-UTR in the eukaryotic selenoprotein mRNA has several advantages. First, there is no restriction on the amino acid sequence to maintain the SECIS stem-loop structure when the SECIS is not in the ORF. Secondly, the process of unfolding and refolding the SECIS stem-loop is not needed during the translation. Finally, the flexibility of the SECIS as such is much higher, and the guidance of multiple Sec insertions from the same mRNA is also allowed [104; 119; 120; 121].

When Sec is synthesized in the eukaryotes, the Ser moiety on Ser-tRNA$^{Sec}$ needs to be phosphorylated by O-phosphoseryl-tRNA$^{Sec}$ kinase (PSTK) first, followed by the conversion from phosphoseryl-tRNA$^{Sec}$ to Sec-tRNA$^{Sec}$ catalyzed by eukaryotic selenocysteine synthase (SecS) [122]. The selenium donor in eukaryote (and in bacteria) is selenophosphate, converted from selenide by selenophosphate synthetase 2 (SPS2) using ATP as the co-factor. There is another selenophosphate synthetase 1 (SPS1), which has been described in eukaryotes [123]. However SPS1 is not required for selenoprotein synthesis. Interestingly, SPS2 itself is also a selenoprotein containing an active site Sec and is essential for selenoprotein synthesis [60; 124].

In bacteria, the connection between Sec-tRNA$^{Sec}$ and SECIS is dependent on a solo factor SelB. The corresponding function in eukaryote, however, is achieved by two distinct proteins. One is a dedicated Sec-specific elongation factor (eEFSec) that is responsible for delivering the Sec-tRNA$^{Sec}$ to the ribosomal A site for Sec incorporation. But, unlike SelB in bacteria, eEFSec is unable to bind the SECIS element directly. The recognition of eukaryotic SECIS requires the second factor, i.e. SECIS binding protein (SBP2) [125]. The interaction between eEFSec and SBP2 is yet to be clarified, however it seems that the connection is accomplished by the C-terminal domain from both proteins.

In addition to the aspects mentioned above, there are still very many factors involved in this complex biological process of selenoprotein biosynthesis. The mechanism has certainly not yet been completely unraveled. More details can be found in these reviews [40; 103; 104].

### 1.5.3 Selenoprotein expression in archaea

As the phylogenetic tree of life suggested, archaea is closer to eukaryote than bacteria, which, in a way, could also be substantiated by the fact that the archaea selenoprotein synthesis machinery is very similar to that of eukaryote. The SECIS element of the archaea selenoprotein mRNA is located in the mRNA's 3'-UTR. A specific elongation factor (aSelB) is employed to recognize Sec-tRNA$^{Sec}$ and deliver the Sec into the growing polypeptide [31; 39]. However, aSelB is unable to directly bind the SECIS element, indicating that another protein, like SBP2 in eukaryote, is involved in the connection between SECIS and aSelB, although this protein remains unknown [39].

### 1.5.4 Recombinant selenoprotein production in *E. coli*

The heterologous expression of recombinant protein, especially in *E. coli*, has been vastly developed and utilized in almost every aspect of life science since the recombinant DNA technique was invented some 40 years ago. Yet, for a very long time, the unique species-specific selenoprotein synthesis machinery has hindered the development of recombinant selenoprotein productions using conventional recombinant technology. In the late 1990's however, Arnér and coworkers had developed a unique methodology for recombinant selenoprotein production and successfully implemented heterologous expression of mammalian TrxR in *E. coli* [110], which has provided a lot of possibilities in the selenoprotein research field.

As previously mentioned, the SECIS element in bacterial selenoprotein mRNA is situated next to the Sec-encoding UGA codon, while mammalian SECIS element is located in the 3'-UTR of the selenoprotein mRNA. This difference makes direct expression of mammalian selenoprotein in *E. coli*, without altering the wild type amino acid sequence, impossible. However, due to the fact that the Sec of mammalian TrxR1 is penultimately positioned at the very C-terminus of the protein, Arnér and coworkers were able to equip TrxR1 ORF with a bacterial type SECIS element next to the UGA codon without interfering with the coding region of TrxR1, and consequently achieved the heterologous expression of the native rat TrxR1 in *E. coli*. In this study, a modified *E. coli* FDH-H SECIS element was utilized to meet the requirement for the rat TrxR1 sequence as well as the binding site recognized by *E. coli* SelB. Subsequently, an assistant plasmid termed pSUABC harboring three *sel* genes (*selA*, *selB* and *selC*) essential for *E. coli* selenoprotein synthesis constructed in August Böck's lab was introduced into this novel selenoprotein synthesis construct [126], which had increased the

efficacy of selenoprotein production [110; 111; 127; 128]. Using rat TrxR1 as a model for selenoprotein production, Rengby and coworkers further optimized the expression conditions to yield about 40 mg rat TrxR1 with a specific activity of 15-25 U/mg from one liter bacterial culture [129]. The specific activity can be increased to 40-60 U/mg by affinity chromatography utilizing an in-house affinity column named phenylarsine oxide (PAO) sepharose (see section 2.2.3) [88; 130].

The limit of this methodology is that only a few types of natural selenoproteins, mainly mammalian TrxRs, have been expressed. The inherent barrier of the bacterial selenoprotein biosynthesis machinery is still difficult to utilize in other natural selenoprotein expressions, especially for the selenoproteins containing internal or multiple Sec residues.

### 1.5.5 Development of the Sel-tag

As mentioned previously, mammalian TrxR1 is a selenoprotein containing a Sec residue within the C-terminal tetrapeptide -Gly-Cys-Sec-Gly-COOH, which is essential for the catalytic activity acting as redox active site. Inspired by the success of heterologous expression of rat TrxR1 in *E. coli*, a Sel-tag concept was developed for recombinant proteins, mimicking the C-terminal motif of TrxR1 [127]. The Sel-tag can be coupled to recombinant proteins expressed in *E. coli* and used for novel biochemical and biomedical applications. To summarize, the Sel-tag is a redox active motif whose reactivity can be "released" under controlled conditions by reduction with DL-dithiothreitol (DTT) or other reductants. The selenolate of the reduced Sel-tag provides a "handle" that can be utilized for selenium based applications, including a one-step purification procedure based upon the affinity binding to PAO sepharose and residue-specific selenolate-targeted protein labeling with either fluorescent or radioactive ligands. In its oxidized state, however, the selenenylsulfide of the Sel-tag is inert and thereby protects the reactive Sec residue from unspecific reactions with electrophilic compounds [127; 128; 131].

## 1.6 Molecular imaging

### 1.6.1 General introduction

An official definition of molecular imaging has been adopted by the Society of Nuclear Medicine (SNM) and its Molecular Imaging Center of Excellence (MICoE). It reads: "Molecular imaging is the visualization, characterization, and measurement of

biological processes at the molecular and cellular levels in humans and other living systems." [132; 133] Molecular imaging is a broad field that encompasses many techniques, with potential applications ranging from bench to clinical research and has been especially used in oncological, neurological, psychiatric and cardiovascular disciplines. Apart from its obvious diagnostic capability, this technique has been contributing more and more to therapeutic monitoring in recent years by evaluating or optimizing new drugs in both pre-clinical and clinical trials.

Molecular imaging utilizes the chemical interactions of so-called biomarkers, also known as probes or tracers, to visualize particular targets or pathways in a given area of interest. This is very different from other techniques such as X-radiation imaging (X-ray), computed tomography (CT) and magnetic resonance tomography (MRT) which primarily show body structure by detecting the differences in quantities or contrasts such as density or water content.

Among the many different types of biomarkers that have been used in functional imaging, protein-based probes would be ideal for developing molecular imaging techniques based on the specific binding of proteins to unique cell surface receptors, antibody-like binding to specific antigens, and many more. Although proteins possess a high capacity and specificity in target binding, they have yet to be widely used as probes for functional *in vivo* imaging due to the lack of efficient labeling procedures. The innovative Sel-tag technology, however, presents a novel strategy for addressing this dilemma. Through its chemical features, the Sel-tag can be used to achieve specific and residue-selective protein labeling with either fluorescent or radioactive electrophiles and the products can subsequently be used as efficient protein probes for functional molecular imaging. Applications in a few such research areas will be briefly discussed in the following sub-sections.

### 1.6.2 Protein fluorescent labeling

Fluorescent labeling can be generally summarized as a process of covalently or non-covalently coupling a fluorophore to another molecule, mostly a protein or nucleic acid. In most cases, fluorescent labeling of a protein is accomplished by covalently binding amine- or thiol-groups of the side chains of lysine or cysteine residues with a reactive fluorophore. Fluorescein is one of the most common fluorophores used in life science [134]. Others include rhodamine, cyanine, coumarin, etc. and new generations with better biochemical properties are continuously being developed. These

fluorophores are normally conjugated with a reactive group in order to achieve specific labeling of the amine- and thiol-groups. Common reactive groups include amine-reactive isothiocyanate or succinimidyl esters, and thiol-reactive maleimide or iodoacetamide. A few commonly used fluorescein derivatives are shown in Figure 9. These fluorescent compounds are widely used for labeling antibodies and other protein-based probes for use in fluorescence microscopy, flow cytometry and immunofluorescence-based assays such as western blotting and enzyme-linked immunosorbent assay (ELISA).

A protein can also be fluorescently labeled by the recombinant introduction of a self-fluorescent protein as a fusion tag. The most commonly used is green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* [135]. As of today, many new GFP derivatives have been engineered that exhibit different biochemical and physical properties, such as brightness, excitation and emission spectra, photostability, pH resistance, etc. For an in-depth review of these types of proteins and their applications in molecular imaging, see references [136; 137].

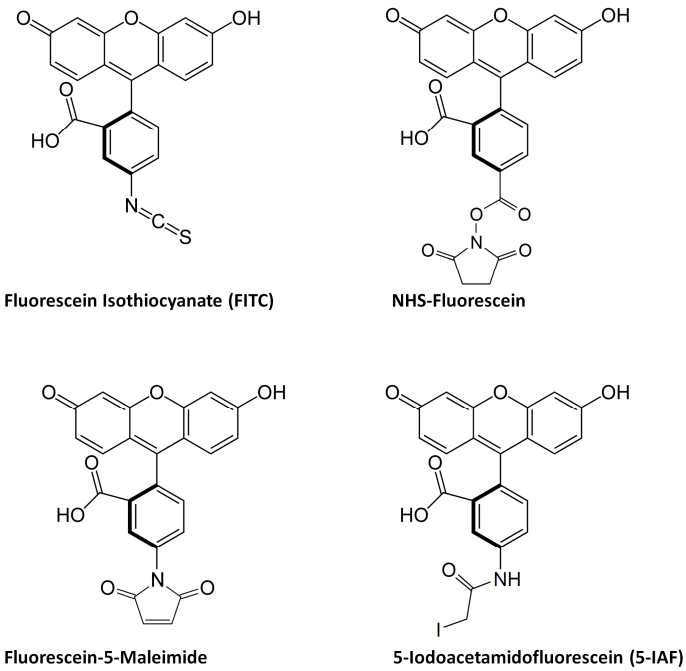



**Figure 9. Fluorescein derivatives.** Fluorescein isothiocyanate (FITC) and NHS-fluorescein are amine-reactive derivatives of the fluorescein dyes, while fluorescein-5-maleimide and 5-Iodoacetamidofluorescein (5-IAF) are thiol-reactive.

In this thesis, thiol-reactive fluorescent compounds such as 5-IAF are utilized in Sel-tagged protein fluorescent labeling. As mentioned previously, the reduced selenolate form of Sec in the Sel-tag is much more reactive than a Cys residue and this feature enables rapid specific Sec-targeted protein labeling in the presence of an excess amount of DTT [127; 128].

### 1.6.3 Protein radiolabeling

Similar to fluorescent labeling, proteins can also be radiolabeled and these radiolabeled proteins are becoming increasingly important in a variety of pre-clinical and clinical investigation.

Sulfur-35 and iodine-125 or iodine-131 are the radionuclides that have mostly commonly been used for direct peptide or protein radiolabeling. Sulfur-35 decays into chlorine-35 by beta emission. Metabolic labeling of proteins with [$^{35}$S]-Met or [$^{35}$S]-Cys has been widely used to study protein synthesis, processing, and primary sequences [138]. Sulfur-35-labeled agents targeting the primary amino group of lysine residues have also been used for radiolabeling proteins [139].

Iodine-125 decays into tellurium-125 by electron capture, producing gamma rays, while iodine-131 decays into xenon-131 with beta and gamma emissions. Both radionuclides can be incorporated into peptide or protein by the mean of direct radioiodination [140]. $^{125/131}I^-$ is converted to the reactive species of $^{125/131}I_2$ or $^{125/131}I^+$ by oxidizing agents and the reactive species can substitute into the tyrosine residues of the protein or into other residues such as histidine and tryptophan in certain circumstances [141].

In the selenoprotein research field, as well as in this thesis, selenium-75 has been routinely used to detect the incorporation of Sec. This will be discussed in more detail in section 2.2.4.

Some of the radionuclides used for functional *in vivo* molecular imaging applications such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) are listed in Table 1. The SPECT nuclides have been used more than PET nuclides for protein labeling, though widely efficient and specific methods are still lacking.

**Table 1. Radionuclides used for PET/SPECT imaging**

| Radionuclide | T½ | Emission | Modality | Examples of clinically used tracers | Ref |
|---|---|---|---|---|---|
| $^{15}O$ | 2.03 min | $\beta^+$ | PET | $^{15}O$-$H_2O$ | 142 |
| $^{13}N$ | 9.97 min | $\beta^+$ | PET | $^{13}N$-$N_2$, $^{13}N$-ammonia | 143; 144 |
| $^{11}C$ | 20.38 min | $\beta^+$ | PET | $^{11}C$-choline, $^{11}C$-methionine | 145; 146 |
| $^{68}Ga$ | 67.71 min | $\beta^+$, $\gamma$ | PET | $^{68}Ga$-DOTATOC | 147 |
| $^{18}F$ | 109.7 min | $\beta^+$ | PET | $^{18}FDG$, $^{18}FES$ | 133; 148 |
| $^{64}Cu$ | 12.7 hr | $\beta^+$, $\gamma$ | PET | $^{64}Cu$-ATSM | 149 |
| $^{76}Br$ | 16.2 hr | $\beta^+$, $\gamma$ | PET | $^{76}Br$-MIBG | 150 |
| $^{124}I$ | 4.18 days | $\beta^+$, $\gamma$ | PET | $^{124}I$-cG250 | 151 |
| $^{99m}Tc$ | 6 hr | $\gamma$ | SPECT | $^{99m}Tc$-Anx A5, $^{99m}Tc$- sestamibi | 152; 153 |
| $^{123}I$ | 13.3 hr | $\gamma$ | SPECT | $^{123}I$-FP-CIT | 154 |
| $^{111}In$ | 2.8 days | $\gamma$ | SPECT | $^{111}In$-DTPA trastuzumab | 155 |
| $^{67}Ga$ | 3.3 days | $\gamma$ | SPECT | $^{67}Ga$-citrate | 156 |

### 1.6.4 Positron Emission Tomography

PET is a non-invasive nuclear medicine imaging technique, which produces 3D images of functional processes in the body as traced by molecules labeled with positron emitting radionuclides (Table 1). In the radioactive decay, a positron is emitted, it travels a few millimeters (depending on the media and positron energy), slows down eventually to collide with an electron and they annihilate each other to produce energy in the form of two anti-parallel 511 keV gamma-rays. Hence it is possible to localize their source along a straight line of coincidence when the gamma-rays are captured by two detectors in a PET camera ring [157]. The most commonly used positron emitting nuclides include $^{15}O$, $^{13}N$, $^{11}C$ and $^{18}F$, while others such as $^{64}Cu$, $^{124}I$, $^{76}Br$, $^{82}Rb$ and $^{68}Ga$ are used, but less often [158]. By varying the tracer molecules, PET imaging is used in many basic and clinical research areas, for example, cell proliferation can be observed by targeting DNA replication using positron-emitting thymidine analogues as the tracers [159]; bone remodeling can be studied using [$^{18}F$]fluoride ion [160]; tumor perfusion can be estimated using [$^{15}O$]$H_2O$ [161].

Currently the positron emitting tracer most widely used clinically is [$^{18}F$]fluorodeoxyglucose (FDG) [162]. It is a D-glucose analog in which the 2-hydroxyl group has been replaced by a $^{18}F$ (figure 10). After the injection into the blood stream, FDG can be specifically transported into cells by glucose transporters and subsequently enter the first step of glycolysis. FDG is phosphorylated to FDG-6-phosphate by hexokinase, but the product cannot serve as a substrate for the next step in glycolysis due to the lack of 2-hydroxyl group. As a result, FDG-6-phosphate accumulates in the cell. Therefore, FDG is acting as a tracer predominantly targeting glucose transporters

and/or hexokinase, which means higher metabolic demands for glucose will be result in higher FDG accumulation. FDG/PET is often used in cancer diagnoses because many types of cancer cells have increased glucose metabolism. The accumulation of FDG in cancer cells may therefore be higher than in "background" tissues, which allows their localization. However, FDG uptake is neither selective nor specific for cancer cells. FDG/PET is also regularly used to assess alterations of glucose metabolism in the brain, in cardiovascular diseases, Alzheimer's disease and other central nervous system disorders, infections, autoimmune and inflammatory diseases [163; 164; 165; 166; 167].

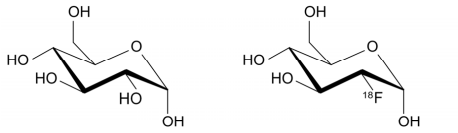



**Figure 10. Comparison of D-glucose and [$^{18}$F]FDG.**

Dedicated small-animal PET cameras have been becoming more widely available in recent years allowing the use of rodents and rabbits [168] as experimental subjects, which can greatly facilitate the development of molecular imaging assays prior to human tests. The spatial resolution of these dedicated animal scanners is higher than that of clinical scanners (1~2 mm *vs* 3~8 mm, respectively). However, small animals can only be injected with correspondingly small masses of tracer molecules, which therefore requires high specific radioactivity of the tracers used in small animal imaging [158].

The lack of anatomical information in PET imaging has recently been compensated by combining it with other morphology-based techniques, such as CT and MRI. PET/CT systems are already the clinical routine worldwide, since the information obtained simultaneously from both modalities significantly improves diagnoses and decision making [169; 170]. PET/MRI, however, is still at the prototype level, continuously undergoing development and refinement [171]. Future technical advances are expected to narrow the current gap between the PET component of PET/MRI devices and stand-alone PET scanners as well as the PET component form PET/CT system in terms of sensitivity and spatial resolution [172].

In summary, the development and improvement of PET technologies as well as novel tracers will be important for taking full advantage of the high sensitivity and efficacy of PET as a diagnostic imaging modality.

# 2 PRESENT INVESTIGATION

## 2.1 Aims of this thesis

This thesis has two major focuses both centered on studies on mammalian TrxR. First, inspired from our successful heterologous expression of rat TrxR1 in *E. coli*, we have developed the concept of Sel-tag by mimicking the C-terminal redox active tetrapeptide motif of TrxR1, which could be coupled to recombinant protein expressed in *E. coli* for novel applications in both biochemistry and biomedicine. Secondly, bearing its natural "Sel-tag", recombinant rat TrxR1 can be further purified over an in-house PAO sepharose to greatly enrich the Sec containing form (i.e the active enzyme), which allows us to crystallize this wild type selenoenzyme and solve the structure and in-depth studies of enzymatic kinetics on TrxR1 and its mutants were subsequently performed. More specifically, the aims for each of the papers are as followed:

**Paper I:** In this study, we characterized the necessity of Sec for the Sel-tag reactivity and evaluated the Sel-tag based purification and fluorescent- or radiolabeling of recombinant proteins produced in *E. coli*.

**Paper II:** In this study, we utilized Sel-tag in an *in vivo* system to investigate the mechanism of antigen presentation by carbohydrate based particles (CBP). This was achieved by tracking the selenium-75 labeled Sel-tagged Fel d 1, the major cat allergen, in a mouse model.

**Paper III:** In this study, we evaluated the potential of a Sel-tag in functional imaging by multimodality imaging of apoptosis through the Sel-tagged annexin A5 utilized in both fluorescent based optical imaging and PET imaging.

**Paper IV:** In this study, we crystallized wild type rat TrxR1 and solved the structure. The structural information about Sec and its interaction with its surrounding environment, together with the studies on several rat TrxR1 mutants, gave us a better understanding of the catalysis of this enzyme.

**Paper V:** In this study, we have observed an unusual phenomenon that TrxR1, normally serving as an antioxidant enzyme, is able to catalyze a significant production of superoxide through an inherent oxidase activity. Some of the superoxide may subsequently be converted to free hydroxyl radicals. This study was mainly accomplished through electron spin resonance (ESR) using wild type rat TrxR1 and several mutants.

## 2.2 Comments on methodology

The methods used in **Papers I-V** can be found in the "Material and Methods" sections from each paper respectively. In addition, the methods related to Sel-tag technology can also be found in the paper included in this thesis as the **Appendix**, which includes:

- Principles of the design and construction of a Sel-tagged protein
- Production of PAO sepharose
- Sel-tagged protein purification through the PAO sepharose
- *In situ* selenium-75 labeling of the Sel-tagged protein
- Sec-specific fluorescence labeling
- Sec-specific labeling with a positron emitting electrophile

A few comments of the methodologies utilized in this thesis will be briefly described in the following text, as they may be of interest for some readers of this thesis.

### 2.2.1 Recombinant selenoprotein production in *E. coli*

As mentioned earlier, the method we used for selenoprotein production in *E. coli* is only suitable for the selenoproteins whose Sec is incorporated in the C-terminal region if any amino acid sequence translated from bacterial SECIS is not supposed to be introduced. There are several unique features about the bacterial FDH-F SECIS element we have used in the thesis. It contains an invariable loop region that binds the Sec-specific SelB elongation factor and it has to be positioned 11 nucleotides (nt) downstream from the Sec-encoding UGA codon (see section 1.3.3). When these requirements are fulfilled, the Sec residue can thereby be introduced into the protein located from the ultimate to preantepenultimate position in the protein (Figure 11).

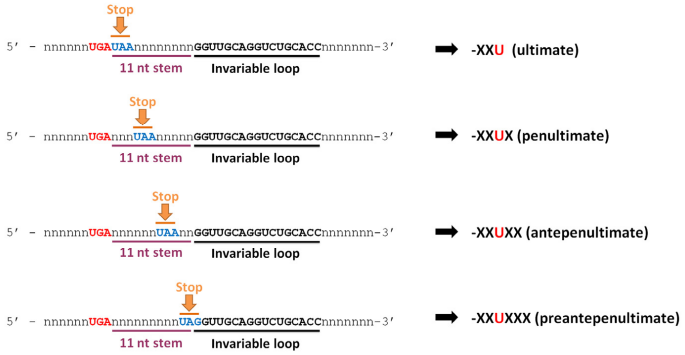


**Figure 11. Potential position of Sec in the recombinant selenoprotein produced in *E. coli*.** The UGA codon has to be positioned 11 nt upstream of the invariable sequence of the FDH-F SECIS element. The real stop codon is indicated by the arrow. Translated sequences of the Sec-containing protein C-terminal are on the right.

### 2.2.2 Application of Sel-tag

To our knowledge, no other protein probes have been reported that are as versatile as the Sel-tag utilizations in biotechnological applications in terms of specific protein purification and protein labeling. However the application of Sel-tag does require a few prerequisites for the protein of interest to be Sel-tagged. First of all, it must be possible to produce the target protein in *E. coli*. Secondly, the function or activity of the target protein must not rely on its carboxy-terminal since the Sel-tag can only be introduced at the C-terminus of the protein. Finally, the target protein must tolerate reduction with DTT or other equivalent reductants if the reactive selenolate form of Sec is needed for the downstream application. However for applications such as those utilizing selenium-75 labeled proteins, DTT is not necessarily a concern.

### 2.2.3 Protein purification through PAO sepharose

PAO sepharose was originally developed for purification of proteins containing vicinal dithiols [173; 174]. We reasoned that the Sel-tag should also have a high affinity, if not better, toward PAO sepharose, which was proved to be correct (Figure 12) [127; 128; 131]. This affinity could be further substantiated by the fact that PAO is an efficient inhibitor of mammalian TrxR1 specifically targeting the enzyme's C-terminal active site [100].

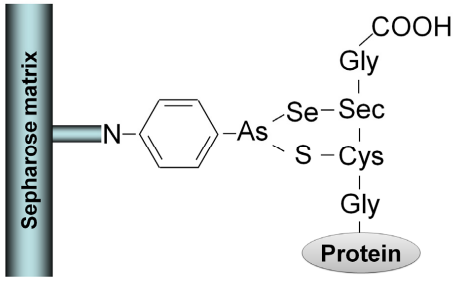



**Figure 12. Schematic illustration of the affinity between PAO sepharose and Sel-tagged protein including mammalian TrxR.** To bind to PAO sepharose, the Sel-tag motif needs to be reduced by DTT. Eventually the covalently bound protein can be eluted by DMPS (2,3-dimercapto-1-propanesulfonic acid, sodium salt) [128].

There was an affinity chromatography product using the same principle called ThioBond that was commercially available from Invitrogen. However, it was discontinued. As a result, we synthesize PAO sepharose ourselves nowadays for our TrxR or Sel-tagged protein purification. The principle of making PAO sepharose is to couple *p*-aminophenylarsine oxide (PAPAO) to the sepharose harboring pre-activated carboxyl group, so the primary amine group from PAPAO is covalently attached to the carboxyl group by forming a peptide bond. Both precursors are available from different vendors, however, we also synthesize PAPAO ourselves using a method previously described [175].

### 2.2.4 *In situ* selenium-75 protein labeling

Selenium-75 labeling is an easy method for detecting selenoproteins. In our lab, this method was routinely used to confirm the integrity of the recombinant selenoprotein expression system that has been constructed. To achieve this, [$^{75}$Se]-selenite is added to the selenoprotein expression bacterial culture to enable the *de novo* synthesis of [$^{75}$Se]-Sec using [$^{75}$Se]-selenite as the selenium source. Consequently, the [$^{75}$Se]-Sec will be incorporated into the nascent selenoprotein given all conditions and components for selenoprotein production are provided (see section 1.3.3). In addition to [$^{75}$Se]-selenite (0.5-1 μCi/mL bacterial culture), we normally add an additional 5 μM normal selenite into the culture to ensure a sufficient selenium supply. For studies requiring a higher specific activity [$^{75}$Se]-labeled protein such as [$^{75}$Se]-Fel d 1 used in **Paper II**, more [$^{75}$Se]-selenite up to 15 μCi/mL could be added [176; 177].

Another important issue in increasing the efficiency of Sec incorporation is the timing of the selenoprotein expression induction. Rengby and coworkers found it is favorable to add IPTG into the bacterial culture at its late exponential phase. The so-called "24, 24, 2.4" protocol [129] is utilized throughout this thesis in the production of rat TrxR1 as well as all other Sel-tagged proteins.

### 2.2.5 Selenolate-targeted protein labeling

Most, if not all, thiol-reactive reagents are expected to have a higher affinity to a selenolate. However, they are called thiol-reactive reagents for a reason. When Sel-tag is utilized for protein labeling, we should always keep in mind that if there is a Cys residue in the protein that is vital to the function of the protein, it should not be targeted. We minimize the risk of Cys-targeting by three measures, namely using lower

pH, short time, and excessive DTT upon protein labeling through Sel-tag. We normally label Sel-tagged protein with electrophilic compounds, such as 5-IAF and Alexa488-meilamide in PBS (phosphate buffered saline, pH 7.4) or TE buffer (50 mM Tris-HCl, 2 mM EDTA, pH 7.5). Sec is more reactive than Cys due to the fact that the selenol group is mostly deprotonated at physiological pH while the thiol group is mostly protonated due to the different pKa values (5.2 for Sec, 8.3 for Cys). The reaction is normally very rapid, lasting for 20 minutes, and is terminated with desalting (e.g NAP-5 column), which efficiently minimizes Cys targeting. Quick labeling is also a prominent advantage of Sel-tag when short-lived positron emitting electrophiles such as [$^{11}$C]methyl iodide are used for labeling. Last but not least, during Sel-tag labeling, we normally have 10-20 fold molar excess of DTT compared to the target protein, which could also protect any structural Cys from unwanted alkylation.

### 2.2.6 Endotoxin contamination

This thesis is largely dependent on recombinant protein production in *E. coli*. Therefore the endotoxin contamination in the protein preparation must be taken into consideration when *in vivo* imaging is performed utilizing these protein samples.

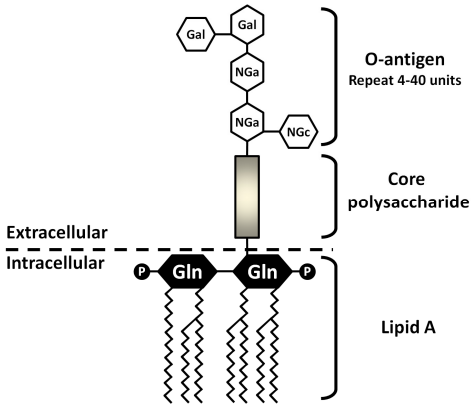



**Figure 13. Schematic structure of *E. coli* LPS.** Gal, galactose; NGa, N-acetyl-galactosamine; NGC, N-acetyl-glucosamine; Gln, Glucosamine. Adapted from Magalhaes (2007) [178].

Endotoxin is mainly related with Gram negative bacteria such as *E. coli* and it almost exclusively refers to lipopolysaccharides (LPS, Figure 13), which consists of a polysaccharide chain and a lipid moiety (lipid A) [179; 180]. Generally, the toxicity of LPS

is associated with lipid A while the immunogenicity is associated with the polysaccharide components. Being temperature and pH stable molecules, the removal of endotoxins from labile biomolecules, such as proteins, is very challenging. The most common methods for endotoxins removal are ultrafiltration and ion exchange chromatography. Endotoxins are approximately 10 kDa in size but can form large aggregates [181], which makes ultrafiltration a good method for removing endotoxins from low molecular weight compounds. However, this method is inefficient for endotoxins removal from protein samples due to their relatively similar sizes. With ion exchange chromatography, it is only effective to remove endotoxins from positively charged proteins since endotoxins are normally negatively charged due to the phosphate groups [182]. However, positively charged proteins and negatively charged endotoxins may form complexes which can reduce the overall efficiency of endotoxin removal. Polymyxin B affinity chromatography can also be utilized for removing endotoxins since polymyxin B, a peptide antibiotic, has a very high affinity toward the lipid A moiety of most endotoxins. Methods for endotoxin removal have been reviewed in some detail recently [178].

Currently, the most used test for endotoxin levels is the Limulus Amebocyte Lysate (LAL) assay, also known as the Bacterial Endotoxin Test, and the endotoxin limits are expressed as EU (endotoxin units) [183]. In preclinical research using animal models, the endotoxin limit is derived from human dose of 5 EU per kg and the maximum endotoxin level considered safe for a 30-g mouse is 0.15 EU administered over a 1-hour period [184].

### 2.2.7 Preparation of recombinant rat TrxR1

As mentioned earlier, the heterologous selenoprotein expression in *E. coli* inevitably results in a mixture of two forms of the target protein, a full length Sec containing selenoprotein and a UGA-truncated protein. For TrxR1, the truncated version (tTrxR1) is devoid of Sec-dependent enzymatic activity and the specific activity solely dependent on full length version (FL-TrxR1) of the recombinant TrxR1 becomes decreased. In **Paper IV**, rat TrxR1 prepared for crystallization was purified through two affinity chromatography (i.e. 2', 5'-ADP sepharose and PAO sepharose), of which 2', 5'-ADP sepharose was used to pull down both tTrxR1 and FL-TrxR1 from the crude bacterial lysate while PAO sepharose was used to remove tTrxR1 from the FL-TrxR1. A typical purification process could increase the specific activity of TrxR1

from 15-20 U/mg prior to PAO sepharose purification to 40-50 U/mg afterwards. For a very long time we believed that this preparation of TrxR1 with around 40 U/mg specific activity should be mainly existed as the full length form, because the specific activity of wild type TrxR1 obtained from direct purification from rat liver was reported to be around 35 U/mg [185]. However, our recent mass spectrometry analysis on different batches of TrxR1 has surprisingly shown that even for a TrxR1 preparation having a specific activity as high as 53 U/mg, only about 65% of the total area of all peaks detected are designated to FL-TrxR1 (Figure 14) [130]. In this paper we also suggested that, theoretically, rat TrxR1 with full Sec content should have a specific activity of 70-80 U/mg.

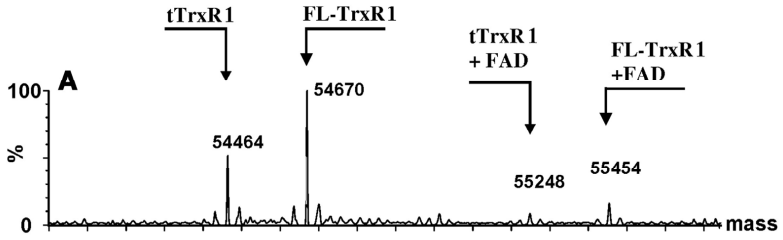



**Figure 14. Mass spectrometry analysis of Sec-enriched TrxR1 from PAO Sepharose purification [130].** TrxR1 was denatured using 50% acetonitrile and analyzed with TOFMS and the deconvoluted zero-charged ions (average mass) are shown. Peaks of 54,670±2 Da correlate with the theoretical mass of FL-TrxR1(54,671 Da) and peaks of 54,464±2 Da correlate with the theoretical mass of tTrxR1 (54,646 Da). Peaks of 55,250±2 Da correspond to tTrxR1 with bound FAD and peaks of 55,454±2 Da correspond to FL-TrxR1 with bound FAD.

Another issue particularly important for recombinant TrxR1 preparation is the contamination from endogenous *E. coli* glutathione reductase (GR). Like mammalian TrxR1, GR also belongs to the family of homodimeric pyridine nucleotide-disulfide oxidoreductases and can bind to 2'5'-ADP sepharose specifically. As a result, the *E. coli* GR is often co-purified with recombinant TrxR1 after the 2'5'-ADP sepharose purification. For most studies on TrxR, this trace amount of contamination of GR is acceptable, but for some enzymatic studies, especially involved with the Grx system, this GR contamination could become crucial. For that reason it is important to remove *E. coli* GR. We currently employ two approaches to solve this problem. One is to include PAO sepharose purification in the TrxR1 preparation procedure since GR has

no affinity to this column, and the other is to use a special GR-deficient BL21 (DE3) gor$^-$ *E. coli* strain for rat TrxR1 production.

### 2.2.8 Electron spin resonance

In this thesis, particularly in **Paper V**, electron spin resonance (ESR) spectroscopy was intensively used to study the inherent NADPH oxidase activity of mammalian TrxR1.

ESR is a technique for studying chemical species that have unpaired electrons, such as organic and inorganic free radicals. Unlike NMR detecting the excited spins of atomic nuclei, ESR detects excited electron spins instead. Although both techniques share similar physical concepts, ESR is less widely used than NMR because most stable molecules don't possess unpaired electrons. However, this also means that the ESR technique is extremely specific, since there won't be any background ESR spectra from the ordinary chemical solvents and matrixes.

Since radicals are very reactive and short lived, normally they do not occur in high concentrations in biological environments. Specialized chemicals called spin trapping reagents are used for ESR measurements by extending the lifetime of the radicals via formation of spin adducts that yield unique ESR spectra (figure 15). In **Paper V**, several spin trapping reagents were used, including 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) and α-phenyl-*N*-*tert*-butylnitrone (PBN).

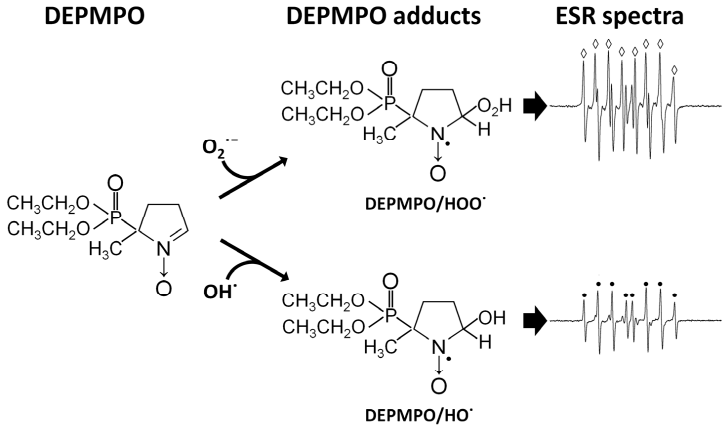


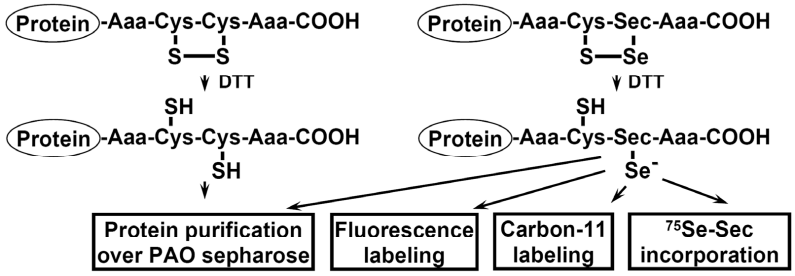

**Figure 15. Radical detection by ESR.** DEPMPO is one of the most common spin trapping reagents for detection of superoxide and hydroxyl radical. Both radicals can attack DEPMPO to form the DEPMPO adducts, which have a longer half life and yield unique spectra under ESR study.

## 2.3 Results and discussion

### 2.3.1 Paper I

**Selenolthiol and dithiol C-terminal tetrapeptide motifs for one-step purification and labeling of recombinant proteins produced in *E. coli*.**

*(Scheme: Illustration of dithiol and selenolthiol tag applications)*




As previously described, we have shown that a redox-active Sec-containing tetrapeptide Sel-tag (Gly-Cys-Sec-Gly) can be used as a fusion motif for recombinant proteins produced in *E. coli*.

Here, we have further developed the Sel-tag motif based on prior results of using mutants of *Drosophila melanogaster* TrxR. That enzyme has a cysteine residue in place of selenocysteine which is activated by flanking serine residues [186]. Thus, we have produced recombinant Fel d 1 [187], the major cat allergen from the domestic cat (*Felis domesticus*), with two dithiol (-GCCG and -SCCS) and two selenolthiol (-GCUG and -SCUS) C-terminal tags. We subsequently assessed the use of these tags in one step purification, fluorescent labeling, and positron emitter labeling.

PAO sepharose could be utilized to efficiently purify all four Fel d 1 variants from whole bacterial lysates in a single purification step yielding apparently homogeneous protein as judged by coomassie-stained SDS-PAGE analysis. The yield was about 5-10 mg pure protein from 1 liter bacterial culture. We next found that all tags could be fluorescent labeled with 5-IAF, with -GCCG being the least reactive and -SCUS being the easiest to label. We finally assessed the use of both the selenolthiol and dithiol tags for selective labeling with a positron emitter typically used for positron emission tomography (PET) studies. For this, [$^{11}$C]$CH_3I$ was utilized. Similar to the fluorescent labeling, both the selenolthiol and dithiol variants could be radiolabeled

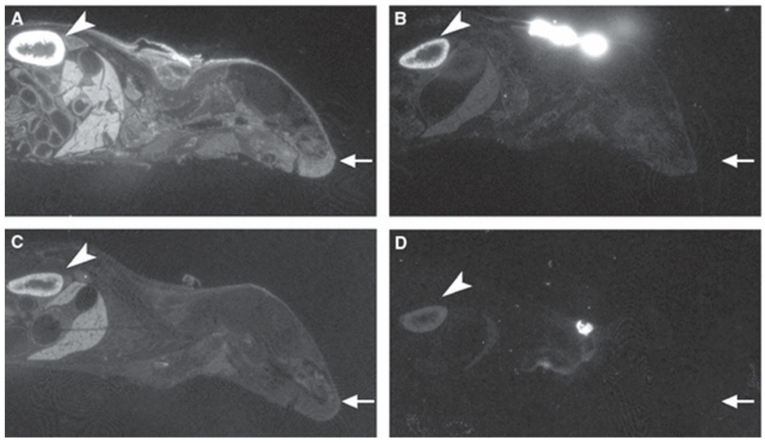
with carbon-11. The labeling efficiencies of the selenolthiol motifs were, however, about 2~3-fold higher than the dithiol variants. In addition, only the selenolthiol tags can be labeled with selenium-75 through the *in situ* metabolic labeling.

In summary, these results illustrate that the use of a Sel-tag or derivatives thereof for purification and residue-specific radiolabeling constitutes a promising technique for a wide range of novel protein-based applications mostly based upon Sec targeting with electrophilic compounds.

### 2.3.2 Paper II

**Prolonged antigen-exposure with carbohydrate particle based vaccination prevents allergic immune responses in sensitized mice**

*(Figure: Whole-body autoradiography tracking of [$^{75}$Se]rFel d 1)*




Mice are widely used for *in vivo* models of allergy and, in most cases, a functional animal model has to be established individually for each and every allergen due to the lack of a standard method to be applied. In this study, we have developed a mouse model for studying the major cat allergen Fel d 1[187] and part of the goal is to use selenium-75 labeled Sel-tagged Fel d 1 in this animal model in order to detect and track the allergen *in vivo*. This was an approach previously demonstrated successfully for another allergen, Der p 2 [188].

To mimic pathogens and enable effective phagocytosis and antigen presentation, a small adjuvant is often used. In this study, we have utilized a novel carbohydrate based particle (CBP), sized 2 μm, as an adjuvant to be coupled with recombinant Fel d 1 (rFel d 1). The vaccination with coupled CBP-rFel d 1 in rFel d 1-sensitized mice after allergen challenge prevented eosinophilic infiltration and airway inflammation. We have also observed that CBP-rFel d 1 vaccination could induce rapid antibody production even after just one injection, compared to either non coupled rFel d 1, CBP alone, or a simple mixture of both. This rapid induction of allergen-specific IgG suggests a potentially efficient treatment using CBP for allergy.

Utilizing FITC-labeled CBP, we found the CBP remained at the injection site at least 5 days after subcutaneous administration, which suggested that it would be possible to use CBP coupled allergen to extend the antigen presentation time. Whole-body autoradiography using $^{75}$Se-labeled rFel d 1 coupled with and without CBP has substantiated this hypothesis. Briefly, we have performed an *in vivo* tracking of 100 μg [$^{75}$Se]rFel d 1 (2 μCi) that was either coupled to CBP or adsorbed to aluminum hydroxide (alum). These two types of allergen complexes were subcutaneously injected into the four model mice (2 for each allergen complex) respectively. Twenty-four hours or one week after the injection, the mice were sacrificed and frozen for tape sectioning (60 μm thick). The sections were subsequently placed on X-ray film for autoradiography.

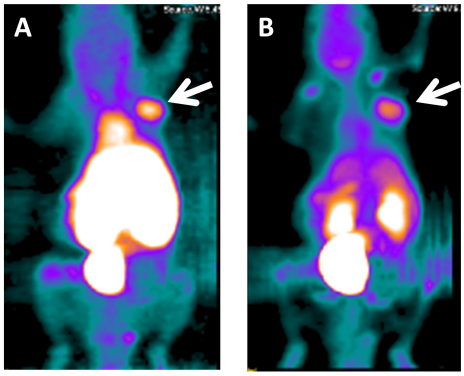
The radioactivity in mice receiving [$^{75}$Se]rFel d 1 adsorbed to alum was spread throughout the animals after 24 hours and remained detectable after 1 week. However, the radioactivity in CBP-[$^{75}$Se]rFel d 1 immunized mice was located almost entirely at the injection site after 24 hours and the injection site was still detectable 1 week after the injection.

We believe these observations have further strengthened the concept that CBP could be utilized to improve antigen-depot effects and therefore become a good adjuvant candidate for allergy vaccination both in a prophylactic and therapeutic perspective.

### 2.3.3 Paper III

**Multimodality imaging of Apoptosis through Sel-tagged Annexin A5**

*(Figure: Sequential PET imaging of xenograft tumor in the same mouse using A: [$^{11}$C]-AnxA5 and B: [$^{18}$F]FDG. Tumor is indicated by arrow)*




Annexin A5 is a member of the annexin family that share the property of $Ca^{2+}$-dependent binding to negatively charged phospholipids [189]. Programmed cell death (PCD), or apoptosis, is characterized by a series of morphological and biochemical changes, including exposure of negatively charged phosphatidylserine (PS) on the cell surface [190; 191; 192]. Through its binding with PS, annexin A5 has been successfully used to detect apoptotic cells both *in vitro* and *in vivo* [193]. In this study, we have equipped annexin A5 with the Sel-tag and subsequently labeled this protein through the Sel-tag with either fluorescent 5-IAF or [$^{11}$C]methyl iodide. The fluorescent- or radio-labeled annexin A5 retains its functionality for specific binding to apoptotic cells and proved to be useful in either fluorescent imaging or PET imaging.

Using 5-IAF labeled Sel-tagged annexin A5, we are able to image either apoptotic or necrotic HeLa cells induced by the treatment of cisplatin. We could identify, through flow cytometry analysis, myeloma cells undergoing cell death treated with Velcade®. These results show that fluorescently labeled Sel-tagged annexin A5 maintains functional binding to apoptotic cells.

Carbon-11 is one of the most important positron emitters for PET studies, yet due to its fairly short half life, it is very difficult to perform *in vivo* PET imaging of a protein probe using carbon-11 as the positron emitter. In this study, we have further

optimized the protocol for protein radiolabeling with [$^{11}$C]$CH_3I$ through the Sel-tag (see Materials and Methods section in the paper). The tracer preparation takes about 45 minutes from the end of cyclotron bombardment to the end of synthesis and the radioactive conversion from [$^{11}$C]$CH_3I$ to [$^{11}$C]-AnxA5 is about 10-20% (decay-corrected). HPLC analysis demonstrated the tracer was sufficiently radiochemically pure for *in vivo* studies. Due to the short half-life of carbon-11, we were able to perform two consecutive PET imaging in the same animal in the same day, including two sequential [$^{11}$C]-AnxA5 in studies of liver apoptosis or two different tracers (i.e [$^{11}$C]-AnxA5 and [$^{18}$F]FDG, in tumor-bearing mice).

Using [$^{11}$C]-AnxA5, we imaged massive liver apoptosis induced by anti-FAS mAb in mice through two different experimental procedures. In the first one, two mice were intravenously injected with 10 μg anti-FAS mAb for 2 or 3 hours. [$^{11}$C]-AnxA5 was then intravenously injected followed by an immediate one-hour PET scanning. In untreated mice, the majority of the radioactivity was in the kidney and bladder by the end of a 60 min scan, showing extensive urinary elimination. In contrast, in the treated mice radioactivity accumulated in the livers and showed increased exposure to the anti-FAS mAb. In another experiment, two consecutive PET scans were performed in the same animal, prior to and after the 10 μg anti-FAS mAb treatment respectively, enabling each animal to be its own control. A marked increase of the radioactivity uptake in the mouse liver was observed after the antibody treatment and the pharmacokinetics is clearly different.

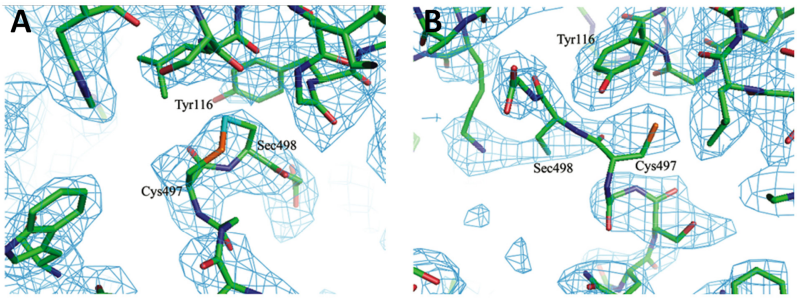
In a tumor PET imaging model, the xenograft tumors developed from FaDu cells could also be visualized with both [$^{11}$C]-AnxA5 and [$^{18}$F]FDG. The pharmacokinetics, however, was different for the two tracers. The *ex vivo* analysis by phosphorimaging of the excised tumor was performed immediately following the FDG scan and the higher resolution phosphorimaging was in line with the rather homogeneous distribution of radioactivity observed within the tumor in the PET images. Studies are continuing in the usefulness of [$^{11}$C]-AnxA5 to monitor responses to chemotherapeutic agents *in vivo*.

To conclude, we believe that the Sel-tag concept can be applied in broad aspects of molecular imaging based upon the utilization of protein probes. With the selenium-dependent chemical features of the Sel-tag, it becomes possible to use proteins as efficient probes for functional imaging using PET as well as many other imaging applications.

### 2.3.4 Paper IV

**Crystal structure and catalysis of the selenoprotein thioredoxin reductase 1**

*(Figure: 2Fo-Fc electron density map showing the model of rat TrxR1 C-terminal active site in either oxidized selenolthiol (A) or reduced selenenylsulfide (B) form.)*




Prior to this paper, several structural studies on mammalian TrxR have been published. However, none of these studies were able to use the wild type Sec containing enzyme that we have utilized here. This is mainly due to the limited availability of this pure selenoenzyme.

In this study, we have used more than 200 mg of rat TrxR1 having a specific activity of at least 40 U/mg for numerous crystallization attempts. Crystals with reasonable resolution were obtained in the presence of 10 mM $NADP^+$ from a solution of 15% PEG3350, 12% ethylene glycol, and 0.1 M HEPES, pH 7.5. The final models of TxR1 had a good geometry and consisted of 3 dimers with a high quality electron density map. The last six residues, where the Sec was located, appeared to be very flexible as we expected. However, weak electron density for the C-terminal motif was observed for three subunits. This allows us to define the general direction of the chain and, most importantly, we could determine the position and the structure of the selenenylsulfide motif in the oxidized enzyme at 3.1 Å resolution (PDB code 3EAO). The structure of the reduced enzyme containing a selenolthiol motif was also determined at a resolution of 2.75 Å (PDB code 3EAN), although the modeling is slightly poorer than the oxidized form. We also believe the reduced selenolthiol motif in the 2.75 Å structure was due to radiolysis.

Surprisingly, the C-terminal oxidized selenenylsulfide motif was located far from the N-terminal redox active site, where it is believed to be the electron donor for the C-

terminal active site. The selenium of Sec-498 in the selenenylsulfide bond was packed against the Tyr-116 phenol ring. However, in the reduced state it turned toward solvent and the presumed Trx-binding site of the enzyme, whereas the sulfur of Cys-497 retained a position below the Tyr-116 ring. Based upon this structural information, we have proposed that the TrxR1-Trx intermediary complex exists in a mixed selenenylsulfide between Cys-32 of Trx and Sec-498 of TrxR1, which could readily be built with only very minor shifts in the active site cleft upon regularization. Upon interaction between the N-terminal active site, our model suggested that a selenenylsulfide intermediate between Sec-498 and Cys-59 was formed during the reduction of the C-terminal active site. However, we are well aware that the flexible C-terminal could also allow modeling a Cys-497 to Cys-59 disulfide intermediate.

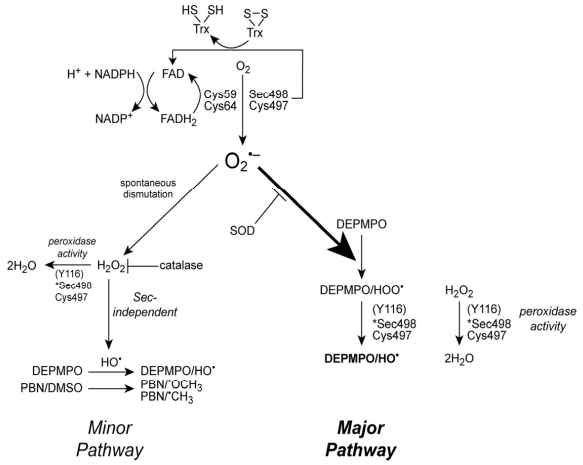
Tyr-116 became an interesting target for this study due to its close position to the C-terminal active site. We therefore constructed three mutants, i.e. Y116F, Y116I, and Y116T. The Y116F was not soluble while Y116I and Y116T were obtained in high yields. All the enzymes including wt TrxR1 and the two Y116 mutants were purified as Sec-enriched proteins through PAO sepharose. For all the substrates we used, including DTNB, selenite, Juglone, lipoamide and Trx, both Y116I and Y116T were catalytically active with a lower enzymatic efficiency as indicated by their lower $k_{cat}/K_m$ ratio for all the substrates tested herein compared with wild type enzyme. However Y116I displayed a surprisingly 7-fold elevated turnover utilizing juglone as substrate than with wild type TrxR1, which suggested that Tyr-116 mutations may not only affect the catalytic efficacy of TrxR1 but also potentially divert electron flow to advantage for some substrates, such as juglone. Briefly, we believe that the substitution of Try-116 has diminished the attack on Sec-498 of the selenylsulfide from Cys-59. Thus the C-terminal is left in the inactive oxidized state, while the N-terminal active site is preferably in its active conformation containing a reactive Cys-59 thiolate and a charge transfer complex between FAD and Cys-64. These are thereby available for other types of reactions, including one- (or two-) electron reduction of quinones such as juglone.

Based on the structural and kinetic data, we have proposed a model to explain the catalysis of mammalian TrxR1 and the effects of Tyr-116. Although we believe this model is the most plausible given our current knowledge of this enzyme and it is in agreement with many prior studies, we are well aware that the model should be corroborated by future studies and may need to be revised.

### 2.3.5 Paper V

**The selenium-independent inherent pro-oxidant NADPH oxidase activity of mammalian TrxR and its selenium-dependent direct peroxidase activities.**

*(Scheme: Inherent NADPH oxidase activity of TrxR1 and source of the resulting ESR findings.)*




As a key component of the thioredoxin (Trx) system, mammalian thioredoxin reductase (TrxR) is a Sec-containing flavoenzyme that has disulfide reductase activity using Trx as the principal substrate. The antioxidant functions of TrxR/Trx are widely accepted. However, TrxR may also have pro-oxidant properties especially upon redox cycling with certain low molecular weight substrates such as 2, 4-DNCB, curcumin, and juglone. Here, we studied these effects in more detail using novel rat TrxR1 mutants and Electron Spin Resonance (ESR) with 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) as the spin trapping agent. In principle, the large amount of TrxR catalyzed $O_2^{\cdot -}$ generation stimulated by the agents mentioned above could be detected by the adrenochrome or cytochrome *c* methods. However, these methods are not sensitive enough to detect small amounts of $O_2^{\cdot -}$. ESR with DEPMPO is ~20-fold more sensitive than the cytochrome *c* method for detection of $O_2^{\cdot -}$, which allowed us to examine the significant inherent NADPH oxidase activity of mammalian TrxR in the absence of certain redox cycling agents. Also, as described earlier, mammalian TrxR is an enzyme containing multiple active sites. Therefore to clarify the contribution of TrxR catalyzed $O_2^{\cdot -}$ generation from these different active sites, we have used wild

type rat TrxR1 as well as its seven mutants in this study, which includes three previously established mutants, i.e. Y116I, Y116T (**Paper IV**) and a truncated mutant that is missing Sec-498 and Gly-499 [194], and four new TrxR1 mutants, namely three single mutants (C59S, C64S, and U498C) and a double mutant (C59S/C64S).

TrxR clearly exhibited enhanced NADPH oxidase activity and could generate free radicals including both hydroxyl radicals ($HO^{\cdot}$) and superoxide ($O_2^{\cdot -}$) upon NADPH reduction in the absence of other substrates. For example, 20 μM selenite decreased the ESR signal by 55% and 5 μM Trx together with 100 μM insulin decrease the ESR signal by 56%. The most likely explanation is that electron flow is more preferably channeled to these substrates instead of reacting with $O_2$.

SOD (Superoxide dismutase) and its mimetic MnTBAP (manganese (III) tetrakis (4-benzoic acid) porphyrin chloride) could eliminate both DEPMPO/$HOO^{\cdot}$ and DEPMPO/$HO^{\cdot}$ signals, indicating the $O_2^{\cdot -}$ is required for the generation of the DEPMPO/$HO^{\cdot}$ adduct by TrxR. Typical $HO^{\cdot}$ generation is through the one-electron reduction of $H_2O_2$ by certain metals such as in the Fenton reaction. However the study had shown that free $H_2O_2$ was not required for $HO^{\cdot}$ generation by TrxR.

In this study, we also tried several classic inhibitors of TrxR, including auranofin, cisplatin, and 2, 4-DNCB to investigate their potential effects on the enzyme behavior in term of free radical generation. Auranofin (4 μM) only decreased the DEPMPO/$HO^{\cdot}$ adduct signal by 28% although >99% DTNB-based TrxR activity was lost. Cisplatin (0.1 mM) did not affect the ESR signal much, implying that TrxR still generates DEPMPO/$HO^{\cdot}$ when cisplatin is bound to the enzyme, which agrees with previous reports of cisplatin-derivatized TrxR in the form of SecTRAPs (selenium-compromised thioredoxin reductase-derived apoptotic protein) [194]. 2, 4-DNCB dramatically changed the ESR spectra, with DEPMPO/$HO^{\cdot}$ decreased by 46% and DEPMPO/$HOO^{\cdot}$ increased by 11-fold. The increase in $O_2^{\cdot -}$ reflects the previously reported induction of NADPH oxidase activity by 2, 4-DNCB, with most of the $O_2^{\cdot -}$ generated by redox cycling of the nitro group of 2, 4-DNCB [93].

Closer analyses using different mutants had revealed that the DEPMPO/$HOO^{\cdot -}$ adduct was a direct substrate of TrxR that could be reduced to DEPMPO/$HO^{\cdot -}$ in a Sec-dependent manner. The $O_2^{\cdot -}$ production itself, however, was Sec independent.

All in all, the results presented in this paper probed the details of the catalytic cycle and reductive capacity of TrxR and may have relevance for the diverse reactions catalyzed by TrxR1 within the cellular context.

# 3 CONCLUSION

The major conclusions from each individual paper were as follows:

**Paper I:** Both dithiol and selenolthiol tetrapeptide tags can be used for efficient recombinant proteins purification over PAO sepharose. However, the selenolthiol variants were highly reactive in rapid labeling with electrophilic compounds including positron emitter such as $[^{11}C]CH_3I$. In addition, the selenolthiol variants also permit methodologically simple *in situ* $^{75}$Se-radiolabeling through the incorporation of $[^{75}Se]$Sec.

**Paper II:** The major cat allergen Fel d 1 was radiolabeled with γ-emitting selenium-75 through a Sel-tag and subsequently used in an *in vivo* mouse model in order to track this protein allergen through a whole body autoradiography. From an immunology point of view, the paper has demonstrated that CBP is a potential adjuvant for use in allergy vaccination.

**Paper III:** By equipping a recombinant protein with a novel multifunctional Sel-tag, this opens several new possibilities for purification as well as functional multimodality imaging, including visualization of apoptosis using either fluorescent- or radio- labeled Sel-tagged annexin A5.

**Paper IV:** The structural characteristics of the unique selenenylsulfide/selenolthiol motif of rat TrxR1 were illustrated and a detailed model for its involvement in the enzymatic catalysis, invoking a key role for the aromatic ring of Tyr-116 was proposed.

**Paper V:** Upon NADPH reduction, TrxR1 could generate free radicals resulting in predominant DEPMPO/$HO^{\cdot}$ and some DEPMPO/$HOO^{\cdot}$. However, DEPMPO/$HO^{\cdot}$ formation is dependent upon $O_2^{\cdot -}$ generation due to a Sec-independent NADPH oxidase activity and subsequent Sec-dependant peroxidase-like activity of TrxR reducing DEPMPO/$HOO^{\cdot}$ to DEPMPO/$HO^{\cdot -}$.

# 4 FUTURE PERSPECTIVES

## 4.1 Recombinant selenoprotein production

Since the first selenoprotein was discovered in 1973, new types of selenoproteins have been continuously identified. In recent years, the *in silico* genomics approaches to look for selenoprotein genes based upon searching the unique features of selenoprotein biosynthesis machineries have greatly increased our knowledge about the distribution of selenoproteins among different organisms. It is generally believed that Sec often serves as a catalyst in redox reactions by selenoprotein. However, due to the distinct barriers of selenoprotein synthesis machineries across different species, it is difficult to study these selenoproteins in their wild type Sec-containing form using conventional recombinant protein expression system, especially in *E. coli* as the suppliers of selenoproteins. However, for recombinant expression of certain types of selenoproteins, namely the proteins containing a C-terminal Sec residue, it is possible to utilize the method we employed in this thesis. For other selenoproteins containing internal or multiple Sec, technical barriers remain to this day. Other methods had been utilized to express selenoprotein, such as native chemical ligation [195], recombinant expression in mammalian cells [196], or intein based selenoprotein production [197; 198]. However, all of these methods are neither efficient, nor applicable for production of most selenoproteins.

We have also proposed two approaches for the recombinant expression of selenoproteins containing internal Sec, both are based upon our previous studies of herterologous selenoprotein expression in *E. coli*.

In the first approach, we attempt to utilize an intein methodology, which has a different perspective from the previous method. Briefly, intein is a protein segment that is able to excise itself from its parent protein and rejoin the remaining two polypeptides (N-extein and C-extein respectively) with a peptide bond. In our case, the Sec will be positioned at the C-terminal of N-extein, while the amino sequence derived from SECIS element will be placed in the N-terminal intein. The self removal of intein will result in a complete selenoprotein without traces of the SECIS element. The biggest challenge is to find a suitable SECIS element that is dual-functional. On one hand it should be recognized by the bacterial Sec-specific elongation factor SelB to ensure the Sec incorporation, and on the other hand the mutation inevitably introduced into the engineered intein motif from the SECIS element should not affect its splicing function.

The second one is a directed-evolutionary approach and it still largely remains at a theoretical level. As mentioned earlier, the bacterial Sec-specific elongation factor SelB is composed of two domains, SelB-N and SelB-C. Of these SelB-N is homologous to EF-Tu, which specifically recruits Sec-tRNA$^{Sec}$ and delivers Sec into the growing polypeptide, while the SelB-C is responsible for recognizing the SECIS element. From structural studies, these two domains are relatively independent to each other. Thereby we hypothesized if we can randomly mutate the SelB-C domain, especially at the region for SECIS recognition, we might be able to obtain some Sel-B mutants that could recognize a given sequence (ultimately any given sequence) other than the wild type SECIS as a novel "SECIS" element. To achieve this, a powerful selection method must be employed simultaneously.

We believe both approaches are not trivial and the development of a proof-of-principle is certainly needed.

### 4.2 Biotechnological application based upon selenoprotein

As a proof of principle, we have already shown that we can use Sel-tag for specific protein labeling and subsequently utilize the labeled protein for different applications, such as an *in vivo* tracking of the protein allergen Fel d 1 in a mouse allergen model (**Paper II)** and multimodality imaging of apoptosis through Sel-tagged annexin A5 (**Paper III)**. Ultimately, the major goal of utilizing Sel-tag is to develop and apply new technology for functional tumor imaging based upon selective and specific radiolabeling of protein probes through the Sel-tag. Currently, we are developing this concept in three different directions.

*I. PET imaging of apoptosis*

We are continuously developing the use of Sel-tagged annexin A5 in tumor imaging through PET. We wish to use this protein to assess the extent of apoptosis in mouse models of treated cancer (xenograft tumor model with treatment using conventional and novel chemotherapeutic drugs). The already demonstrated ability of the labeled protein to reveal the timing and degree of chemotherapeutic effects has a high potential for translation to the PET imaging of patients. Correlation of *in vivo* apoptosis detection with the appearance of blood markers will provide a solid ground for novel strategies for detecting and optimizing therapeutic responses.

*II. PET imaging of angiogenesis*

Angiomotin is expressed in the endothelium of newly forming vessels, the inhibition of which prevents angiogenesis as shown using animal models. In this project, we have constructed a Sel-tagged scFv (single-chain antibody fragments) against angiomotin, which will be used to perform *in vivo* angiogenesis imaging in a tumor model. These scFv molecules are derived from the same clone as that inhibiting angiogenesis *in vivo*, whereby the biodistribution and pharmacokinetics of the scFv can be evaluated using PET. With the goal of utilizing these technologies for the validation of ongoing angiogenesis as a means of diagnosis of solid tumor formation *in vivo*, we expect that the development of Sel-tagged scFv based projects to have major clinical and pharmaceutical value for therapy validation. Moreover, a possibility exists based upon this project for the development of new functional imaging for assessment of anti-angiogenic or anti-cancer therapy, based upon the *in vivo* imaging of angiogenesis and the termination of such processes by pharmaceutical treatment.

*III. High-affinity binding molecules for specific cancer detection*

To detect specific types of cancer cells by PET imaging, a cell type-specific PET tracer that can bind with high affinity and high specificity to unique cancer cell surface is needed. For this purpose, a unique type of small polypeptides named affibody will be utilized under the Sel-tag concept. Affibody molecules are generated from the Z-domain, a protein scaffold used in combinatorial library selections derived from *staphylococcal* protein A. Affibody molecules are relatively small (~6 kDa) and highly stable, they can be expressed in *E. coli* with high solubility, and most selected affibody molecules have no Cys residues. All these properties make affibody an optimal candidate for protein labeling through a Sel-tag. As a pilot study, we have chosen an affibody that has been shown to target human epidermal growth factor receptor 2 (HER2) overexpressed in breast cancer cells. Our earlier studies have already shown that the Sel-tagged affibody$_{HER2}$ can be labeled with either fluorescent 5-IAF or positron-emitting [$^{11}$C]$CH_3I$. We are currently constructing a follow-up mouse model to apply this protein tracer for PET imaging of breast cancer. Moreover, several other affibody molecules with different cancer cell targets are also under development.

All together, we believe that the Sel-tag technique provides great opportunities for the development of proteins as biomarkers for non-invasive functional imaging,

especially PET imaging, which eventually might prove useful for cancer research as well as clinical practice.

### 4.3 Mammalian thioredoxin reductase

As one of the most important antioxidant enzymes, research potential around mammalian TrxR is tremendous and there are still so many puzzles about this enzyme and related proteins yet to be solved.

In **Paper IV**, we have solved the structure of rat TrxR1 as a native Sec-containing protein. Based on the structural data and the kinetics of several TrxR1 mutants, we have proposed a model to explain the catalysis by TrxR1. The fact that we are able to determine the crystal structure of a recombinant selenoprotein sheds light on possibilities to produce additional Sec-containing selenoproteins similar to TrxR1 studied herein for further detailed molecular and structural studies.

In a recently published paper, we have identified a stable tetrameric form of Sec-containing rat TrxR1 [130]. In order to understand the conformation as well as the potential physiological impact of this tetrameric TrxR1, we have recently crystallized this unique TrxR1 form and an in-depth study on its structure and several corresponding mutants are currently being carried out.

In **Paper V**, we have revealed that TrxR1 had an inherent NADPH oxidase activity that can surprisingly catalyze a significant superoxide production, which is in line with the previously described induction of TrxR1 NADPH oxidase activity by certain low molecular compounds. Redox cycling of these low molecular compounds catalyzed by the N-terminal active site of TrxR1 seems to play a major role in such NADPH oxidase activity. In a current follow-up study, utilizing ESR, we have intensively investigated the interaction between TrxR and certain quinones to understand which of its redox centers are responsible for quinone reduction.

It has been widely accepted in recent years that TrxR1 is a promising anticancer target. Therefore the discovery of novel compounds that could target TrxR1 are of importance for novel cancer drug development. We have recently submitted a paper describing a high throughput assay for simultaneous identification of inhibitor and substrates of TrxR1 in a single-enzyme, dual-purpose assay. We are now expanding this assay in screening a large chemical library containing more than two hundred thousand compounds. After the screening, the compounds with high scores will be further studied for characterization as either an inhibitor and/or a substrate of TrxR1.

# 5 ACKNOWLEDGEMENTS

The work of this thesis was performed at the **Division of Biochemistry, Department of Medical Biochemistry and Biophysics (MBB), Karolinska Institutet**, as well as the **PET Radiochemistry, Neuroradiology Department, Karolinska University Hospital**. I am grateful to the institutions for enabling me to pursue my research in such an excellent environment.

During my last six years working on this thesis, I have received tremendous help and support from numerous people, and I would like to express my deepest gratitude to all of you and in particular the following people.

**Prof. Elias Arnér**, my principle supervisor. I am so grateful that I wrote you that email back in 2004 and got the opportunity coming to Sweden. You are such an inspiration as a scientist, a group leader and a friend. Your enthusiasm about science and life is truly contagious. Thank you for everything!

**Prof. Sharon Stone-Elander**, my co-supervisor. Thank you for bringing me into the "hot" field and I am thrilled to be working with you so closely, I mean you did the work and I stood behind watching you. Your optimistic attitude is so admirable and I will never forget your warm hug whenever I encountered good or bad things.

Elias and Sharon, you two are truly the best supervisors I can ever imagine! Your encouragement, guidance and support from the beginning to the final level make this thesis possible.

**Prof. Arne Holmgren**, thank you for sharing your sharp insights towards science and great comments on my studies. I also enjoy listening to your story about the old day science.

**Ms. Lena Ringdén**, for providing your excellent administrative support and great effort making the lab more comfortable throughout these years.

**Jacek Andrzejewski**, for being so helpful for the daily life in the lab from ordering chemicals to running the steaming fermentor.

**EA group**, all the former and present colleagues, for making a wonderful working environment and being so supportive. In particular, **Dr. Linda Johansson** for excellent initiation works on the Sel-tag and generous help during my first year in Sweden. **Dr. Olle Rengby** for a great accompanier in the office and a great organizer of all the gatherings and parties. It's just fun being with you! **Drs. Tomas Nordman** and **Karin Anestål** for the good time together a few years back. **Dr. Stefanie Prast-Nielsen** and **Sofi Eriksson** for the help in the cell lab and fun time during our group retreats. **Dr. Pascal Dammeyer** for good time both on and off the tennis court. **Marcus Cebula** for being a great roommate and badminton partner, and I always admire your spirit daring to challenge yourself in many respects. **Dr. Jianqiang Xu** for being so humble and helpful in the lab, especially for kindly taking care of my plates and gels during late evening and weekends. **Dr. Victor Croitoru** for being so kind and willing to share your experience from the industry world. **Dr. Hanna-Stina Martinsson Ahlzén** for great enforcement to the PET imaging project, you simply save me tons of

time! **Xiaoxiao Peng** and **William Stafford** for your help and I wish you the best of luck for your studies in this wonderful group.

**SSE group**, it's such a pleasure working with all of you guys! **Dr. Jan-Olov Thorell** for being the "hottest" person who we all rely on. **Erik Samén** for your great computer skills on processing those PET images. And J-O, Erik, what a fun went fishing with you guys. **Dr. Li Lu** for your magic hand on our little patients. And your kindness and encouragement always make me feel so good when I came to the microPET lab. **Emma Jussing** for always having a smile and I enjoyed the good time with you and the rest during our Turku trip. **Dr. Anna Fredriksson** for the early time helping with the protein labeling in the hot lab.

All the former and present members from **AH group**, **Drs Aristi Potamitou-Fernandes**, **Catrine Johansson**, **Malin Fladvad**, **Maria Lönn**, **Laura Papp**, **Jun Lu**, **Jianguo Fang**, **Liangwei Zhong**, **Eng-Hui Chew**, **Mohammad Taghi Goodarzi**, **Johanna Ungerstedt**, **Rajib Sengupta**, **Alexios Vlamis-Gardikas**, **Farnaz Zahedi Avval**, **Isaac Hashemy**, **Javier Avila-Carino**, **Christina Carvalho, Sergio Montano and Tomas Gustafsson**. The German power **Drs. Christopher Horst Lillig**, **Carsten Berndt**, **Christoph Hudemann** as well as **Eva-Maria Hanschmann** and **Lars Bräutigam**. A special thanks to **Drs. Yatao Du** and **Huihui Zhang**, for sharing a great friendship for almost 10 years and all the helps from making dumpling to fixing computer. And **Xu Zhang** for sharing interesting news and tips of life such as the magic Copenhagen diet. All together, you make Biochemistry Division a great place!

All the guest researcher who I closely worked with, **Drs. Oliver Rackham** and **Aleksandra Filipovska**, thank you for sharing your broad knowledge on molecular biology and enzymology. **Dr. Sharon Rozkovsky** for NMR studies on selenoproteins. **Dr. Alexio Capovilla** for the great effort on the HIV vaccine project. In particular I would like to thank you and **Dr Grant Napier** for your hospitality when Elias and I visit you in South Africa. What a great fun in the bush!

All my co-authors, especially **Drs. Guro Gafvelin**, **Sarah Thunberg**, **Theresa Neimert-Andersson** and **Hans Grönlund** for good discussion and practice on the Fel d 1 project. **Prof. Stig Linder**, **Dr. Maria Hägg Olofsson** and **Pádraig D'Arcy** for sharing your knowledge on apoptosis and great input to the Annexin project. **Dr. Tatyana Sandalova** and **Prof. Ylva Lindqvist** for helping me crystallized the wild type TrxR1 and solved the structure. **Prof. Charles Myers** and your group and collaborators from University of Wisconsin for the profound study on TrxR1 utilizing ESR. **Juan Carlos Fierro-González** for being so kind when we were working on TrxR1 from *C. elegans*.

My close collaborators **Helena Wållberg** and **Prof. Stefan Ståhl** from KTH for the project of tumor imaging through affibody. **Nathalie Luna Persson** and **Prof. Lars Holmgren** from CCK for the project of angiogenesis imaging through scFv.

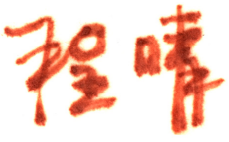
I would like to thank **Ásmundur Oddsson**, **Drs. Masatoshi Nukui**, **Daniel Ågren** and **Robert Schnell** from other units of MBB for your help and friendship. **Chad Tunell** and **Anders Lundsjo** for your excellent IT support. **Jeroen Frijhoff**, **Per-Magnus Eriksson, Erik Ragnerstam, Patryk Sawicki, Arthur Jullien** for the

good time on tennis court. My students **Awad Smew**, **Xiaona Jin**, **Sandra Nilsson**, and **Linnea Ek** for bearing with me and for your great effort on our projects.

I came to Sweden on Aug 25th, 2004 together with **Yuan Xue** and had the first meal in Sweden prepared by **Jinfeng Shen, Jun Ma**, and **Yin Dou**. And since then I have met so many Chinese friends and all of you make this journey full of fun and not so lonely. To name a few more, **Zhe Zhang, Liqun He, Yin Sun, Yu Li, Xiaoda Wang, Ling Zheng, Hao Mo, Hao Wu, Min Wan, Zhijie Xiao, Xiaofeng Zheng, Xin Wang, Likun Du, Jikui Guan, Bin Zhao, Ziming Du, Jingwen Shi, Xun Wang, Jia Mi, Hongshi Qi, Xiaoqun Zhang, Tong Liu, Dadi Niu, Yi Sun, Wenhui Cai, Lanjunzi Chen, Yang Dong, Jinzhi Hu, Liyue Hu, Haining Li, Meng Li, Jiayou Luo, Te Ma, Zhuchun Peng, Yao Shi, Yu Sun, Lijun Tang, Yanling Wang, Ge Wu, Liang Wu, Jie Yan, Haideng Zhang** and so many more. I am sorry I can only mention your name here without sharing a few words on each and every one of you, because if I do that, this acknowledgement will be longer than my introduction. I will thank you in person when I see you. However in particular, I would like to thank **Di Sun** for all the good time we spent together back in Pax and KI, and for exhibiting your courage never giving up. **Yan Wang, Yanhong Zhang,** and **Hua Gao** for the really good time in California. **Xingqi Shi** and **Tangliang Li** for a good trip in Germany. **Xiaorui Cao** for spending all the good time with me back in China, here in Sweden and in Italy. **Yunqian Zhao** and your lovely family for your generous hospitality when I visit Okinawa, and it's such a great pleasure traveling with you. **Ying Tang** for sharing the nice moment in the Pax corridor and on the road to France, Germany, Czech and Norway. **Wen Zhang**, life is full of laughter with you, I appreciate your humor, consideration and being fully confident with me. And I am also very grateful that you "smuggled" so many things from China for me.

**My dearest family**, for all these years, your love and supports are never ended. **Mum**, thank you for giving me life, teaching me how to become a decent man and letting me pursue my dream freely. There is still a long journey ahead of us and I will make both you and Dad proud of me! My brother **Zhiqun**, sister in law **Jianhua** and niece **Ying**; My sister **Hong**, brother in law **Youqian** and nephew **Yucheng**; My cousins **Weiwei**, **Zhigang** and your family, I thank you all for taking good care of me and our family when I am away, and there is no other words can describe how grateful I am. I love you all.

Again, Thank **YOU**!



***"In the Name of Selenium"***
*Signature of CHENG Qing written on the waterman paper soaked with L-Cysteine (50 mg/mL) through a Chinese calligraphic brush using Selenite (5 mM) as ink. The reddish color is revealed through the elemental Selenium reduced from Selenite by L-Cysteine.*

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